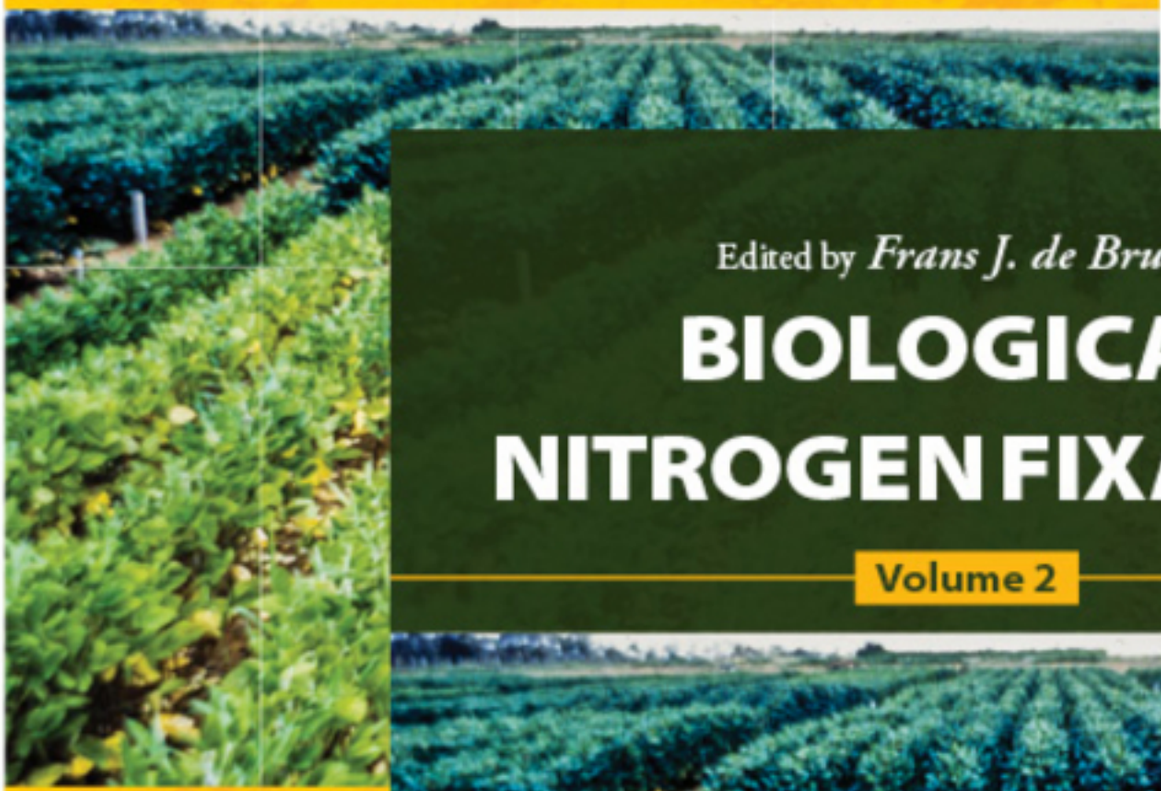
Edited by *Frans J. de Bruijn*

BIOLOGICAL NITROGEN FIXATION

Volume 1



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Volume 2

WILEY Blackwell

Biological Nitrogen Fixation

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Cover photo: Courtesy of Embrapa Cerrados, Iêda C. Mendes and Mariangela Hungria. Soybean plants cultivated with and without inoculation in a first-year Cerrado Oxisol, poor on N in Planaltina, Brazil.

The front plot shows small uninoculated soybean plants, light green and yellowish because of the lack of N and the surrounding plots show large dark green plants inoculated with symbiotic nitrogen fixing *Bradyrhizobium japonicum* bacteria. For further details see Chapter 99 by Hungria and Mendes.

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This work is dedicated to my two daughters, Waverly de Bruijn-Klaw and Vanessa de Bruijn, for their support and interest even from a distance and to my wife, Cathy Senta-Loys de Bruijn, for her love and understanding during the editing of this book.

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Section 10 Infection and Nodule Ontogeny

Preface

Nitrogen is arguably the most important nutrient required by plants, being an essential component of all amino acids and nucleic acids. However, the availability of nitrogen is limited in many soils, and although the earth's atmosphere consists of 78.1% nitrogen gas (N_2), plants are unable to use this form of nitrogen. To compensate this, modern agriculture has been highly reliant on industrial nitrogen fertilizers to achieve maximum crop productivity (Ferguson et al. 2010).

However, a great deal of fossil fuel is required for the production and delivery of nitrogen fertilizer. Indeed, industrial nitrogen fixation alone accounts for 50% of fossil fuel used in agriculture. This can be exceedingly expensive. In recent years, the price of chemical fertilizers has increased dramatically due to rising fossil fuel costs. Moreover, carbon dioxide (CO_2), which is released during fossil fuel combustion, contributes to the greenhouse effect, as does the decomposition of nitrogen fertilizer, which releases nitrous oxide (NO_x), itself about 292 times more active as a greenhouse gas than carbon dioxide (Ferguson et al. 2010). In addition, applying chemical fertilizers is largely inefficient, as 30–50% of applied nitrogen fertilizer is lost to leaching, resulting in significant environmental problems, such as the eutrophication of waterways. Thus, there is a strong need to reduce our reliance on chemical nitrogen fertilizers and instead optimize alternative nitrogen inputs (Ferguson et al. 2010).

Biological nitrogen fixation is one alternative to nitrogen fertilizer. It is carried out by prokaryotes using an enzyme complex called nitrogenase and results in atmospheric N_2 being reduced into a form of nitrogen diazotrophic organisms, and plants are able to use (ammonia; see Introduction). It is this process and its major players, which will be discussed in this *Biological Nitrogen Fixation* book.

The research in the field of Biological Nitrogen Fixation is very active at the moment, especially in the subfield of symbiotic nitrogen fixation. Although a number of books and proceedings of the International Congresses on Nitrogen Fixation and North American Symbiotic Nitrogen Fixation Conferences have appeared during the last 10 and more years, a comprehensive book on the field from biochemistry of nitrogenase, through expression and regulation of nitrogen genes, taxonomy, evolution, and comparative genomics of nitrogen-fixing organisms; their physiology and metabolism; their life in the rhizosphere and under stress conditions, rhizobial "Omics," plant "Omics," nodulation of legumes and nonlegumes, recognition, infection and nodule ontogeny, nitrogen fixation and assimilation, field studies, inoculum preparation and application of Nod factors, endophytic nitrogen fixers, cyanobacteria, and nitrogen fixation and cereals does not presently exist, and this book aims to fill this void.

A number of authors were selected based on the programs of the 21st North American Conference on Symbiotic Nitrogen Fixation in Columbia, Missouri, USA (June 2010); the 17th International Congress on Nitrogen Fixation in Fremantle, Western Australia (December 2011); the 16th Australian Nitrogen Fixation Conference (Manly, June 2012); the programs of the "European Nitrogen Fixation Congress (ENFC)," held in Munich, Germany, from the September 2, 2012, to September 5, 2012; and the satellite meetings on "Genomics of nitrogen fixing bacteria" and the "Symposium on Biological Nitrogen Fixation with Non-legumes" (The latter three were attended by the Editor upon invitation by the Conference Organizer Dr. Anton Hartmann).

The best known and most extensively studied example of biological nitrogen fixation is the symbiotic interaction between nitrogen-fixing "rhizobia" and legume plants. Here, the rhizobia induce the formation of specialized structures ("nodules") on the roots or sometimes stems of the legume plant and fix nitrogen, which is directly assimilated by the host plant; in return, the plant provides the required energy source for the energy-intensive, nitrogen-fixation process. It is this symbiotic interaction, which will be highlighted in this book.

While this book features many chapters on the model system for indeterminate nodule formation, the *Sinorhizobium meliloti–Medicago truncatula* symbiosis, little

information is presented on the *Mesorhizobium loti–Lotus japonicus* symbiotic model system for determinate nodule formation. The reason for this is the simultaneous publication edited by Dr. S. Tabata and Dr. Jens Stougaard (Springer-Verlag) specifically focused on this topic, and the reader is referred to this book for details on this important model symbiotic system.

While legumes are important as major food and feed crops and are the second group of such crops grown worldwide, the first group (cereals such as wheat, maize, and rice) does not have this symbiotic nitrogen-fixing interaction with rhizobia. It has thus been a focus of a number of studies to transfer the ability to fix nitrogen to cereals, and different timely approaches toward this goal are also included in the book. The case of rice will be discussed in the following section.

Rice is the most important staple food for over 2 billion people in Asia and for hundreds of millions in Africa and Latin America. To feed the ever-increasing population of these regions, the world's annual rice production must increase from the present 460 million to 560 million tons by the year 2000 and to 760 million tons by 2020 (Ladha et al. 1997).

If future increase in rice production has to come from the same or even reduced land area, rice productivity must be greatly enhanced to meet these goals. Nitrogen is the nutrient that most frequently limits agricultural production of rice and other cereals. As pointed out earlier, global agriculture now relies heavily on N fertilizers derived at the expense of petroleum. Nitrogen fertilizers, therefore, are expensive inputs costing agriculture more than $45 billion (US) per year (Ladha et al. 1997).

In the tropics, lowland rice yields 2–3.5 ton per hectare, utilizing naturally available N derived from biological nitrogen fixation (BNF) by free-living and plant-associated (endophytic) nitrogen-fixing organisms and from mineralization of soil N. Achieving 50% higher rice yield needed by 2020 will require at least double the 10 million ton of N-fertilizer that is currently used each year for rice production. Manufacturing the fertilizer for today's needs requires 544×10^9 MJ of fossil fuel energy annually (Ladha et al. 1997).

Thus, alternatives to chemical fertilizer must be sought.

BNF for rice and other cereals has been the "holy grail" for decades now and has been explored in laboratories worldwide, mainly looking at free-living, associative, and endophytic nitrogen-fixing organisms. These studies have been presented and discussed at numerous International Nitrogen Fixation Congresses, Nitrogen Fixation with Non-legumes Meetings, and the IRRI funded Working Group Meetings of the Frontier Project on Nitrogen Fixation in Rice and Other Non-Legumes, the latter in the late 1990s (Ladha et al. 1997). Recently, the Bill and Melinda Gates Foundation convened a small meeting on the subject and several projects were funded, including the transfer of the nitrogen-fixation (nitrogenase) genes into cereals and the transfer of the ability to form nitrogen-fixing nodules to cereals. This has greatly stimulated research in this area.

Rice transformation is now routine and since the genome has been sequenced and extensive genetics is available, it should serve as a "model species," while for other cereals, a recently developed *Brachypodium* model system has been established.

The discovery of a "common symbiotic pathway" in arbuscular mycorrhizal, rhizobial, and actinorhizal symbioses and the identification of conserved pathway genes in legumes and monocots (including rice; Venkateshwaran et al. 2013) have made the extension of the ability to fix nitrogen in nodular associations, with cereals as a more realistic endeavor.

The direct transfer of nitrogen fixation (*nif*) genes into nonlegumes has also become more feasible especially since it has been shown that six out of the numerous *nif* genes are absolutely required for FeMo-co biosynthesis and nitrogenase activity, both *in vitro* and *in vivo*.

It is likely that the products of some genes that are required for FeMo-co biosynthesis *in vivo* could be replaced by the activities of plant counterparts.

Thus, we are entering a very promising period of research on BNF, both in more conventional systems and nonlegumes such as rice, based on rapidly advancing basic studies on the chemistry, biochemistry, genetics, physiology, regulation, taxonomy, genomics and metagenomics, and metabolism of nitrogen-fixing organisms (and their hosts). This is the topic of this book, which should be a major resource for scientists in the field, and those wanting to enter it, as well as teachers and agricultural and molecular specialists wanting to apply the technology.

Frans J. de Bruijn

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Chapter 1

Introduction

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Nitrogen fixation is a process by which nitrogen (N_2) in the atmosphere is converted into ammonia (NH_3) (Postgate, 1998). Atmospheric nitrogen or elemental nitrogen (N_2) is relatively inert: it does not easily react with other chemicals to form new compounds. Fixation processes free up the nitrogen atoms from their diatomic form (N_2) to be used in other ways. Nitrogen fixation, natural and synthetic, is essential for all forms of life because nitrogen is required to biosynthesize basic building blocks of plants, animals, and other life forms, for example, nucleotides for DNA and RNA and amino acids for proteins. Therefore, nitrogen fixation is essential for agriculture and for the manufacture of fertilizer (Free Wikipedia Encyclopedia, Biological Nitrogen Fixation; Postgate, 1998).

Nitrogen fixation also refers to other biological conversions of nitrogen, such as its conversion to nitrogen dioxide. Microorganisms that fix nitrogen are bacteria called diazotrophs (Postgate, 1998). Some higher plants and some animals (termites) have formed associations (symbioses) with diazotrophs.

The first nitrogen-fixing microbe to be discovered was *Clostridium pasteurianum* obtained by S. Winogradsky in 1893. The second free-living diazotroph *Azotobacter chroococcum* was first reported by the Dutch microbiologist M.W. Beijerinck in 1901. Symbiotic nitrogen fixation was discovered by the German agronomists H. Hellriegel and H. Willfarth in 1886–1888 (Postgate, 1998).

Biological nitrogen fixation (BNF) occurs when atmospheric nitrogen is converted to ammonia by an enzyme called *nitrogenase* (Postgate, 1998). The reaction for BNF is as follows:

$$N_2 + 8H^+ + 8e^- \rightarrow 2\,NH_3 + H_2$$

The process is coupled to the hydrolysis of 16 equivalents of ATP and is accompanied by the coformation of one molecule of H_2. In free-living diazotrophs, the nitrogenase-generated ammonium is assimilated into glutamate through the glutamine synthetase/glutamate synthase pathway (Postgate, 1998). Enzymes responsible for nitrogenase action are very susceptible to destruction by oxygen. Many bacteria cease production of the enzyme in the presence of oxygen. Many nitrogen-fixing organisms exist only in anaerobic conditions, respiring to draw down oxygen levels or binding the oxygen with a protein such as leghemoglobin (Free Wikipedia Encyclopedia, Biological Nitrogen Fixation; Postgate, 1998).

1.1 FREE-LIVING DIAZOTROPHS

Anaerobes: These are obligate anaerobes that cannot tolerate oxygen even if they are not fixing nitrogen. They live in habitats that are low in oxygen, such as soils and decaying vegetable matter. *Clostridium* is an example. Sulfate-reducing bacteria are important in ocean sediments (e.g., *Desulfovibrio*); some Archean methanogens fix nitrogen in mud and animal intestines, as well as in the deep ocean.

Facultative anaerobes: These species can grow either with or without oxygen, but they only fix nitrogen anaerobically. Often, they respire oxygen as rapidly as it is supplied, keeping the amount of free oxygen low. Examples include *Klebsiella pneumoniae*, *Bacillus polymyxa*, *Bacillus macerans*, and *Escherichia intermedia*.

Aerobes: These species require oxygen to grow, yet their nitrogenase is still debilitated if exposed to oxygen. *Azotobacter vinelandii* is the most studied of these organisms. It uses very high respiration rates and protective compounds

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to prevent oxygen damage. Many other species also reduce the oxygen levels in this way, but with lower respiration rates and lower oxygen tolerance (Free Wikipedia Encyclopedia, Biological Nitrogen Fixation; Postgate, 1998).

Oxygenic photosynthetic bacteria generate oxygen as a by-product of photosynthesis, yet some are able to fix nitrogen as well. These are colonial bacteria that have specialized cells (heterocysts) that lack the oxygen-generating steps of photosynthesis. Examples are *Anabaena cylindrica* and *Nostoc commune*. Other cyanobacteria lack heterocysts and can fix nitrogen only in low light and oxygen levels (e.g., *Plectonema*), or in a diurnal rhythm.

Anoxygenic photosynthetic bacteria do not generate oxygen during photosynthesis, having only a single photosystem that cannot split water. Nitrogenase is expressed under nitrogen limitation. Usually, the expression is regulated via the negative feedback from the produced ammonium ion, but in the absence of N_2, the product is not formed and the by-product H_2 continues unabated (biohydrogen). The example species are *Rhodobacter sphaeroides*, *Rhodopseudomonas palustris*, and *Rhodobacter capsulatus* (Free Wikipedia Encyclopedia, Biological Nitrogen Fixation; Postgate, 1998).

1.2 SYMBIOTIC NITROGEN-FIXING BACTERIA

Rhizobia are Gram-negative with the ability to establish an N_2-fixing symbiosis on legume roots and on the stems of some aquatic legumes. During this interaction, bacteroids, as rhizobia are called in the symbiotic state, are contained in intracellular compartments within a specialized organ, the nodule, where they fix N_2. Similarly, *Frankia*, Gram-positive soil bacteria, induce the formation of nitrogen-fixing nodules in actinorhizal plants (Postgate, 1998).

Plants that contribute to nitrogen fixation include the legume family – Fabaceae – with taxa such as kudzu, clovers, soybeans, alfalfa, lupines, and peanuts. They contain symbiotic bacteria called *Rhizobia* within nodules in their root systems, producing nitrogen compounds that help the plant to grow and compete with other plants. When the plant dies, the fixed nitrogen is released, making it available to other plants and this helps to fertilize the soil. The great majority of legumes have this association, but a few genera (e.g., *Styphnolobium*) do not. In many traditional and organic farming practices, fields are rotated through various types of crops, which usually includes one consisting mainly or entirely of clover, alfalfa, or buckwheat (nonlegume family *Polygonaceae*), which are often referred to as "green manure" (Free Wikipedia Encyclopedia, Biological Nitrogen Fixation).

Legumes also include major food and feed crop species, such as soybean, pea, clover, chickpea, alfalfa, and mung bean. They represent the third largest group of angiosperms and are the second largest group of food and feed crops grown globally. They are cultivated on 12–15% of the available arable land and are responsible for more than 25% of the world's primary crop production with 247 million tons of grain legumes produced annually (European Association for Grain Legume Research, 2007). In addition to food and feed crops, legumes such as soybeans and *Pongamia pinnata* have gathered a great deal of attention as future sustainable biofuel sources because of their high seed oil content (Ferguson et al., 2010).

Although by far the majority of plants able to form nitrogen-fixing root nodules are in the legume family Fabaceae, there are a few exceptions: *Parasponia*, a tropical Celtidaceae, is also able to interact with rhizobia and form nitrogen-fixing nodules. Actinorhizal plants, such as alder and bayberry, can also form nitrogen-fixing nodules, thanks to a symbiotic association with *Frankia* bacteria. These plants belong to 25 genera distributed among 8 plant families. The ability to fix nitrogen is far from universally present in these families. For instance, of 122 genera in the Rosaceae, only 4 genera are capable of fixing nitrogen. All these families belong to the orders Cucurbitales, Fagales, and Rosales, which together with the Fabales form a clade of eurosids. In this clade, Fabales were the first lineage to branch off; thus, the ability to fix nitrogen may be plesiomorphic and subsequently lost in most descendants of the original nitrogen-fixing plant; however, it may be that the basic genetic and physiological requirements were present in an incipient state in the last common ancestors of all these plants, but only evolved to full function in some of them (Free Wikipedia Encyclopedia, Biological Nitrogen Fixation).

1.3 ASSOCIATIVE NITROGEN-FIXING BACTERIA

Associative nitrogen fixers form a group of mutualist systems in which there is some interdependence between the partners though both can grow satisfactorily apart (Postgate, 1998). They involve grasses (Gramineae) principally. The prospect of finding bacteria that would form associative relationships with rice, other cereals, or graminaceous crops has provoked considerable interest in those diazotrophic bacteria that inhabit the rhizosphere of such plants (Postgate, 1998). In recent years, several types have been reported, for example, species of the new genera *Herbaspirillum*, *Gluconobacter*, *Azoarcus*, and *Burkholderia*, as well as diazotrophic species of *Acetobacter* called *Acetobacter diazotrophicus* (Postgate, 1998).

1.4 OUTLINE OF THIS BOOK

In this book, the most recent findings about a variety of free-living, associative, or symbiotic diazotrophs are

covered, nitrogenase(s) and their mechanism of action and regulation are reviewed, and the use of diazotrophs in agriculture is summarized.

The flow of the chapters is as follows: This chapter is an introduction to the book by the editor, giving the background to the field, describing the flow of sections and chapters and highlighting sections, individual contributions, and some future trends. Section 1 contains a number of focus chapters (reviews) introducing the main topics of the book, including nitrogenases and how they work, evolution and taxonomy of nitrogen-fixing organisms, the evolution of *Rhizobium* nodulation, and bioengineering nitrogen acquisition in rice. Section 2 covers selected recent advances in the biochemistry of nitrogenases, including the biosynthesis of the FeMo-cofactor (FeMo-co) subunit of nitrogenase, and conserved amino acid sequence features in MoFe, VFe, and FeFe nitrogenases. Section 3 covers the regulation of nitrogen-fixation genes and nitrogenase itself. The chapters in this section will describe regulatory aspects of several different nitrogen-fixing systems, such as *A. vinelandii*, *R. capsulatus*, *Rhodospirillum rubrum*, *Pseudomonas stutzeri*, and *Rhizobium etli*. Section 4 covers taxonomic and evolutionary features of nitrogen-fixing organisms, including chapters on taxonomy as well as the origin and diversity of *Burkholderia* and other beta-rhizobia; the phylogeny of nodulation and nitrogen-fixation genes in *Bradyrhizobium*; and a global census of nitrogenases and nitrogen-fixation genes. Section 5 covers the genomics of selected nitrogen-fixing organisms and the comparative analysis of their genomes. Also included are a chapter on the transfer of the symbiotic island of *Mesorhizobium loti* and a chapter on software program for pan-genomic analysis. Section 6 covers aspects of the physiology and metabolism of nitrogen-fixing organisms and a chapter on the need for photosynthesis for efficient nitrogen fixation in a rhizobial strain, as well as chapters on cytochrome oxidases, the role of BacA in rhizobia, and the analysis of flagellins in *Rhizobium leguminosarum*. Section 7 contains a number of chapters on the rhizobial life in the rhizosphere of plants, including the effect of plant root exudates, role of quorum sensing and quenching, exopolysaccharides, flavonoids, luminochrome, and the response to various stresses. Section 8 deals with the physiology and regulation of nodulation. Chapters include the root hair as a single cell model for systems biology, two chapters on the conserved genetic program among arbuscular mycorrhizal, actinorhizal, and legume–rhizobial symbiosis, the molecular determinants of nodulation in the *Frankia*/*Discaria* symbiosis and the physiology of nitrogen assimilation in the *Datisca–Frankia* root nodule symbiosis, as well as chapters on the Nod-independent symbiosis in *Aeschynomene*, the role of phosphorus efficiency, the regulation of nodule development by auxin transport, and the *NOOT* mutant of *Medicago truncatula*. Section 9 then initiates a series of chapters on nodulation. This section covers the very early events in nodulation, including putative Nod-factor receptors and signal transduction, early signaling in *Frankia*, the role of ectopyrases and cellulose $CelC_2$ in nodulation, and calcium spiking. Section 10 addresses the infection and nodule ontogeny topics. A multitude of aspects are covered in this section, which are as follows: Ca^{2+} signaling and infection thread formation; the role of hormones in nodulation; the role of a transporter in integrating nutrient and hormone signaling with lateral root growth and nodule development; the role of genes encoding MYB coiled-coil and ERF transcription factors; the dissection of the roles in outer and inner root cell layers of plant genes that control rhizobial infection and nodule ontogeny; the multifaceted role of nitric oxide in nodulation; the role of pectate lyase in root infection; the identification of novel *M. truncatula* genes required for rhizobial invasion and bacteroid differentiation, as well as novel approaches such as RNA-seq; and cortical auxin modeling for nodulation. Section 11 covers the "next" stage in nodule biology, namely, the development of bacteroids required for nitrogen fixation and the proteomic profile of the soybean symbiosome membrane. Section 12 addresses briefly N-assimilation (ammonium transport) in nodules and nodule senescence. In Section 13, several "Omics" applications in rhizobia and *Frankia* (metagenomics, transcriptomics, proteomics, genomics) are discussed, such as the metagenomic analysis of microsymbiont selection by the legume host plant, proteomic profiling of *Rhizobium tropici*, the *Frankia alni* symbiotic transcriptome, a comprehensive survey of the Rhizobiales using high-throughput DNA sequencing and gene-targeted metagenomics of diazotrophs in coastal saline soil. Section 14 does the same with (host) plant genomics, proteomics, and transcriptomics, including chapters on the *M. truncatula* genome, retrotransposon *Tnt1* mutagenesis, leveraging large-scale approaches to dissect legume genomics and the *Rhizobium*–legume symbiosis, databases, and functional genomics of symbiotic nitrogen fixation in legumes. In Section 15, the intricacies of nodule formation and functioning are left behind, and the focus is on nitrogen-fixing cyanobacteria, with studies in the open ocean, requirement of cell wall remodeling and cell differentiation in a cyanobacterium of the order Nostocales, and nitrogen fixation in the oxygenic phototrophic prokaryotes (cyanobacteria): the fight against oxygen. Section 16 deals with diazotrophic plant growth promoting rhizobacteria (PGPR) and nonlegumes. This section begins with a historical overview of PGPR and nonlegumes. Beneficial plants associated with *Burkholderia* species, agronomic applications of *Azospirillum* inoculants, and molecular characterization of the diazotrophic bacterial community in sugarcane are discussed. Moreover, the role of auxin signaling in plant–microbe interactions is presented. Finally, a chapter on how fertilization affects the selection of PGPR by the plant is included, as well as the genetic and

functional characterization of *Paenibacillus riograndensis*, Herbaspirillum attachment to maize, and isolation of novel diazotrophs from sugarcane plants. Section 17 covers field studies, inoculum preparation, and quality and response to stress, such as dessication, evaluation of elite soybean varieties in the field, phase variation in *Azospirillum*, and the application of LCOs to legume and nonlegume seeds.

Section 18 is special and deals with the opportunities for nitrogen fixation in rice and other cereals (see Preface). This "holy grail" has been with us for a long time. The first two chapters explore the present possibilities to "engineer" rice for nitrogen fixation. In the first chapter, the history of the quest for BNF in cereals is reviewed, and associative (endophytic) diazotrophy with grasses and transfer of the legume nodulation and nitrogen-fixation traits to cereals are discussed. In the second chapter, the environmental and economic impacts of biological N_2 fixing cereal crops are discussed. In this chapter, the question is raised "how novel are nodules?" (Markmann and Parniske, 2009). Two major approaches are discussed again: Developing the root nodule symbiosis in cereals and transferring the nitrogenase genes into cereal plants, and finding a proper location for their expression in terms of low free O_2 concentration and sufficient energy supply. These two options and the associative and endophytic nitrogen fixation were already discussed in previous meetings such as those of the Working Group of the IRRI Frontier Project on assessing Opportunities for Nitrogen Fixation in Rice (Opportunities for BNF in Rice and other Non-Legumes (Ladha et al., 1997)), as well as The Quest for Nitrogen Fixation in Rice (Ladha and Reddy, 2000). However, we are in a much more informed state now. Recently, the Bill and Melinda Gates Foundation convened a small meeting on the subject and several projects were funded, including the transfer of the nitrogen-fixation (nitrogenase) genes into cereals, and the transfer of the ability to form nitrogen-fixing nodules to cereals. This has greatly stimulated research in these areas.

In the case of rice transformation, it is now routine and since the genome has been sequenced and extensive genetics is available, it should serve as a "model species," while for other cereals (grasses) a recently developed *Brachypodium* model system has been established (Bevan et al., 2010).

The discovery of a "common symbiotic pathway" in arbuscular mycorrhizal, rhizobial, and actinorhizal symbioses and the identification of conserved pathway genes (CSSP, CSP or SYM pathway) in legumes and monocots (including rice) have made the extension of the ability to fix nitrogen in nodular associations with cereals in a more realistic endeavor (Venkateshwaran et al., 2013; see Chapter 108).

The direct transfer of nitrogen-fixation (*nif*) genes into nonlegumes has also become more feasible especially since it has been shown that six out of the numerous *nif* genes are absolutely required for FeMo-co biosynthesis and nitrogenase activities, both *in vitro* and *in vivo*.

It is likely that the products of some genes that are required for FeMo-co biosynthesis *in vivo* could be replaced by the activities of plant counterparts.

Chapter 110 deals with the conservation of the symbiotic signaling pathway (SYM; see earlier) between legumes and rice and the related functional cross-complementation studies. Chapter 111 describes the ecophysiology of the natural endophytic *Rhizobium*–rice association and the translational assessment of its biofertilization performance in the Nile delta.

Section 19 contains a first chapter on nitrogen fixation and nitrogen recycling in insects. In the second chapter, a protocol for the rapid identification of nodule bacteria with MALDI-TOF mass spectrometry is presented. The last chapter of the book gives a comprehensive review on endophytes in plants.

Thus, we are entering a very promising period of research on BNF, both in more conventional systems and in nonlegumes such as rice and other cereals, based on rapidly advancing basic studies on the chemistry, biochemistry, genetics, physiology, regulation, taxonomy, genomics, and metagenomics, and the metabolism of nitrogen-fixing organisms and their hosts. These are the topics of this book, which should be a major resource for scientists in the field, and those wanting to enter it, as well as teachers and agricultural specialists wanting to apply the technology.

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Section 1

Focus Chapters

Chapter 2

Recent Advances in Understanding Nitrogenases and How They Work

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2.1 INTRODUCTION: TWO TYPES OF NITROGENASES EXIST

There are two very different types of nitrogenases (Smith et al., 2004). First, there are those that constitute the Group-1 nitrogenases. These are the three closely related, but genetically distinct, enzymes: the well-studied molybdenum-based enzyme (Mo-nitrogenase), a vanadium-based enzyme (V-nitrogenase), and an enzyme that contains neither heterometal but relies on iron alone (Fe-nitrogenase). Except for the different heterometal atom (Mo, V, or Fe) present, they are otherwise so similar that they must have arisen from a common ancestor (Fani et al., 2000; Young, 2005). In contrast, Group-2 consists of a single representative, the *Streptomyces thermoautotrophicus* nitrogenase. This enzyme is so different from the Group-1 enzymes that it may well be the result of an evolutionarily independent event (Gadkari, 2004; Ribbe et al., 1997). It is completely insensitive to the presence of O_2, CO, and H_2, all of which are potent inhibitors of nitrogen fixation with the Group-1 nitrogenases, and does not catalyze the reduction of acetylene to ethylene. Its unusual structural and mechanistic properties could hold promise of new insights into the process and possibly new ways of extending the benefits of biological nitrogen fixation in agricultural systems. However, this enzyme has not been under study recently and so there is no progress to report.

2.2 OCCURRENCE AND RELATIONSHIPS AMONG THE GROUP-1 NITROGENASES

All known nitrogen-fixing organisms have the Group-1 Mo-nitrogenase; however, the presence of either or both of the V-nitrogenase and Fe-nitrogenase appears to be completely haphazard. For example, both the free-living *Klebsiella pneumoniae* and the rhizobacteria, which form nodules on the roots of legume plants, have only Mo-nitrogenase, whereas other organisms, for example, *Azotobacter vinelandii*, have all three enzymes. Other combinations also occur, for example, *Azotobacter chroococcum* has the Mo- and V-nitrogenases, but *Rhodobacter capsulatus* has the Mo- and Fe-nitrogenases (Newton, 1993).

The expression of the various nitrogenases depends on the availability of the metal ions (either Mo or V) in the growth environment (Pau, 1994). Whenever Mo is available, only the Mo-nitrogenase (*nif*) genes are expressed and the genes, if present, for the other two nitrogenases (*vnf* and *anf*) are repressed. Similarly, when V is available and Mo is absent, only the *vnf* genes are expressed. If both Mo and V are absent, then just the *anf* genes are expressed and only Fe-nitrogenase is produced. This control by metal availability is physiologically reasonable because Mo-nitrogenase is the most efficient N_2-reduction catalyst and Fe-nitrogenase is the least efficient. However, there are only very few areas

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on Earth where Mo is completely absent, which raises the question of whether these nitrogenases have other functions unrelated to nitrogen fixation.

The relatedness of these three nitrogenases is shown by their ability to cross-complement each other (Emerich and Burris, 1978; Newton, 1993; Pau, 1994). All nitrogenases consist of two component proteins: the Fe protein and the MoFe (VFe or FeFe) protein (Smith et al., 2004). These components can be separately purified, but have no N_2-fixation activity alone (but see later). Either component protein of, say, Mo-nitrogenase from *A. vinelandii* forms an active hybrid nitrogenase when it is mixed with the complementary component protein of the V-nitrogenase from the same organism. However, not all component proteins cross-complement each other; for example, neither component protein of the *A. vinelandii* Fe-nitrogenase forms an active hybrid with the complementary protein from its Mo-nitrogenase or V-nitrogenase. Furthermore, mixtures of the Mo-nitrogenase component proteins from different organisms may not produce active hybrid nitrogenases, a notable example being the Mo-nitrogenase components from the strict anaerobe, *Clostridium pasteurianum*.

Both the origin and evolution of the nitrogen-fixation genes and proteins and the mechanisms involved in shaping the process itself continue to engender considerable debate as do the processes involved in distributing this ability among the relatively few N_2-fixing genera of bacteria and archaea. Its apparent haphazard distribution might reflect either a common ancestral property that was lost randomly during divergent evolution or a more recent example of lateral gene transfer. In fact, genome sequences appear to support the likelihood that a combination of both multiple losses and multiple transfers of the nitrogen-fixation genes has occurred. Whatever the case, the genetic relatedness of the Group-1 nitrogenases, including even the order in which the genes are found in genomes, supports a common ancient ancestry (Young, 2005).

So, which of the Group-1 nitrogenases evolved first? One view cites a pyrites-based catalyst that pre-dated enzymes and which might then have been sequestered by early protein-like materials (Wächtershäuser, 1988). This view would suggest that an Fe-based nitrogenase might be the progenitor from which the other nitrogenases developed. Consideration of the limitations that an early anoxic atmosphere would place on the chemistry and availability of Mo, V, and Fe has also led to the conclusion that the V- and Fe-nitrogenases pre-dated the Mo-nitrogenase (Anbar and Knoll, 2002; Raymond et al., 2004). Under these anoxic conditions, Mo (present as water-insoluble MoS_2) would be unavailable to support N_2 fixation, but Fe would be plentiful (Anbar, 2008). An Fe- (and/or V-) nitrogenase could then well have been the logical choice. A similar conclusion results when the occurrence of Mo/S and W/Se in hydroxylases and oxidoreductases in mesophiles, thermophiles, and hyperthermophiles is analyzed (Newton, 2000). As the hot, anoxic, early-Earth cooled, hyperthermophilic microbes would have appeared first, then thermophiles, and finally mesophiles. Only in mesophiles, after conditions have become cooler and presumably more oxygenated, thus the Mo/S-containing (rather than W/Se-containing) enzymes occur. Now, the oxygenated conditions produce the water-soluble MoO_4^{2-}, while simultaneously depleting the environment of Fe, which would be deposited as water-insoluble FeO(OH), and Mo-based enzymes, including Mo-nitrogenase, would become the norm.

A related suggestion similarly invokes a primitive nitrogenase from which the three Group-1 nitrogenases developed (Postgate and Eady, 1988). This suggestion is based on the observation that, although genetically distinct, the three sets of structural (*nifHDK*, *vnfHDGK*, and *anfHDGK*) genes were likely formed by gene iteration (Fani et al., 2000). Furthermore, the products of five *nif*-specific (*nifMBVUS*) genes are required for the biosynthesis of all three nitrogenases; the heterometal cofactors are transferable among themselves; and all have similar catalytic properties (Newton, 1993). An aboriginal enzyme is also the basis of a somewhat contrasting view of nitrogenase evolution that has its basis in phylogenetic and structural analyses (Boyd et al., 2011). Here, the suggestion is that the V- and Fe-nitrogenases are derived from, and not the ancestors of, Mo-nitrogenase, whose formation is driven by local limited supplies of Mo.

2.3 OVERVIEW OF PROPERTIES OF Mo-NITROGENASE

The Mo-nitrogenases from a variety of bacterial genera exhibit a high level of primary (amino acid) sequence identity. The sequence conservation is particularly high in the regions of the MgATP- and metallocluster-binding sites. The Fe-protein component is a homodimer (encoded by the *nifH* gene) of about 64 kDa molecular mass. A single [4Fe–4S] cluster bridges the two identical subunits, each of which has its own MgATP/MgADP-binding site. The Fe protein is a specific reductant for the MoFe protein. The MoFe protein is an $\alpha_2\beta_2$ heterotetramer (encoded by the *nifDK* genes) of about 230 kDa molecular mass. Each αβ-subunit pair contains one copy of each of two different prosthetic groups, namely, the P cluster and the iron-molybdenum cofactor (or FeMo-cofactor or the M center). The latter serves as the site of substrate binding and reduction (Scott et al., 1990). Unlike the P cluster, which is disrupted when removed from the MoFe protein, the FeMo-cofactor can be extracted essentially intact into a variety of organic solvents (Wink et al., 1989). The isolated prosthetic group is then referred to as FeMoco. Each component protein exhibits an electron paramagnetic resonance (EPR) spectrum when isolated in the presence of sodium dithionite, their so-called

"resting state." These EPR signals have been instrumental in determining the direction of electron flow between the component proteins (Orme-Johnson et al., 1972) and in monitoring many of the catalyzed reactions (see later). Three-dimensional structures of the individual component proteins from both wild-type and mutant bacterial strains and their complexes are available (see later).

The physiologically important nitrogen-fixation reaction is outlined in the following equation. Delivery of eight electrons and eight protons results in the catalyzed reduction of one N_2 to two ammonia molecules together with one H_2 molecule and consumes 16 MgATP molecules. The evolution of one H_2 for each N_2 reduced may be either a mandatory step in the mechanism (Simpson and Burris, 1984) or an as yet unexplained kinetic phenomenon.

$$N_2 + 8\,H^+ + 8\,e^- + 16\,MgATP \rightarrow 2\,NH_3 + H_2 + 16\,MgADP + 16\,P_i$$

In vivo, either ferredoxin or flavodoxin acts as the reductant of the Fe protein, whereas *in vitro*, the artificial reductant, sodium dithionite ($Na_2S_2O_4$), is most often used. The Fe protein accepts only a single electron from sodium dithionite, which is then transferred to the MoFe protein with two molecules of MgATP being hydrolyzed (Seefeldt and Dean, 1997). Other reductants, for example, Ti(III) and flavodoxin hydroquinone, used *in vitro* may transfer two electrons to the Fe protein. When both electrons are transferred in a single step to the MoFe protein, with still only two molecules of MgATP being hydrolyzed, the MgATP consumption rate is halved (Lowery et al., 2006). It remains to be seen if both one-electron and two-electron transfers occur *in vivo*. Both the rate of electron transfer (the flux) and the ratio of electrons transferred to MgATP hydrolyzed are independent of the substrate being reduced.

Wild-type Mo-nitrogenase catalyzes the reduction of many other small molecules (Fig. 2.1). All substrates have the same requirements as N_2 reduction, namely, a supply of MgATP, a low-potential reductant, and an anaerobic environment (Newton and Dilworth, 2011). The most commonly used substrate is acetylene, which is reduced by two electrons to ethylene, in a reaction that accounts for about 95% of the electron flux under a 10% C_2H_2/90% argon atmosphere with the remainder going to H_2 production. When no other substrate is present, all electron flux is used for proton reduction to H_2. In addition, H_2 is a specific inhibitor of N_2 reduction, but does not affect either the reduction of any other substrate or its own evolution. When D_2 is used as the specific N_2-reduction inhibitor, HD is formed in a reaction that has all the requirements of a nitrogenase-catalyzed reaction that is also inhibited by CO (Newton and Dilworth, 2011; Burgess and Lowe, 1996). Also shown is carbon monoxide (CO), a well-studied potent noncompetitive inhibitor of all nitrogenase-catalyzed substrate reductions except for proton reduction to H_2. Recently, CO has also been shown to be a substrate for Mo-nitrogenase (Lee et al., 2010).

2.4 OVERVIEW OF PROPERTIES OF V-NITROGENASE AND Fe-NITROGENASE

These so-called alternative nitrogenases also consist of two protein components (Eady, 1996, 2003; Smith et al., 2004). Each nitrogenase has its specific Fe-protein component. The larger component protein of the V-nitrogenase contains a VFe-cofactor, with a V atom replacing the Mo atom of the Mo-nitrogenase. Similarly, the Fe-nitrogenase has no Mo atom and instead has an FeFe-cofactor in an FeFe protein. The high level of primary sequence identity among the Mo-nitrogenases extends to the V- and Fe-nitrogenases and strongly suggests that all nitrogenases share common structural features and mechanistic similarities. One major difference is that both the VFe protein and FeFe protein have additional δ-subunits. Both were initially reported as $\alpha_2\beta_2\delta_2$ heterohexamers (Eady, 1996, 2003) but recently, using immobilized metal affinity chromatography and His-tagging, the VFe protein was purified as an $\alpha_2\beta_2\delta_4$

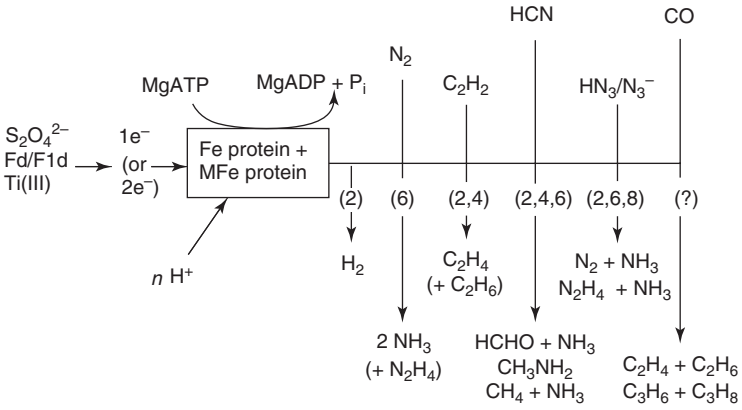

Figure 2.1 Electron donors, substrates, and products of catalysis. Not all products shown are produced by all three Group-1 nitrogenases. CO is not only a substrate but also a potent reversible inhibitor of all nitrogenase-catalyzed substrate reductions except for that of protons to H_2. Fe-p represents the Fe protein, MFe-p is the MoFe/VFe/FeFe protein; reductants are dithionite ($S_2O_4^{2-}$), titanium(III) citrate (Ti(III)), Fld (flavodoxin hydroquinone), or Fd (ferredoxin); numbers in parentheses are the number of electrons used to form the products shown.

heterooctamer (Lee et al., 2009). The δ-subunits apparently bind to the apoprotein during biosynthesis and remain bound thereafter. In contrast, the δ-subunit equivalent of the MoFe protein (encoded by the *nifY* gene) is lost in the late stages of its maturation.

EPR spectroscopy shows that both alternative nitrogenases undergo similar changes to those of the Mo-nitrogenase during turnover. Even so, the dithionite-reduced VFe protein exhibits an EPR spectrum that is considerably more complex, and still not clearly understood, than that of the MoFe protein (Lee et al., 2009), whereas the purified resting-state FeFe protein exhibits no EPR signal (Krahn et al., 2002; Schneider et al., 1997). However, under turnover conditions, the FeFe protein develops a rhombic $S = 1/2$ EPR signal indicating that it is also reduced by electron transfer from its Fe-protein partner (Schneider et al., 1997).

The catalytic properties of both the V- and Fe-nitrogenases also differ in detail from those of Mo-nitrogenase. For N_2 reduction, V- and Fe-nitrogenases use only about 50% and 30%, respectively, of the electron flux to produce NH_3 under 100% N_2 compared to about 75% for Mo-nitrogenase (Dilworth and Eady, 1991; Schneider et al., 1997). V-nitrogenase also produces a very small amount of N_2H_4, which is not observed with Mo-nitrogenase (Dilworth and Eady, 1991). V- and Fe-nitrogenases are also much less effective for C_2H_2 reduction with only about 40% and 15%, respectively, of the flux producing C_2H_4 compared with 95% for Mo-nitrogenase. Again, a small amount of additional product (C_2H_6) not seen with Mo-nitrogenase is formed under a 10% C_2H_2/90% argon atmosphere (Dilworth et al., 1988; Schneider et al., 1997). A recent significant observation is that not only is 10% carbon monoxide (CO) a noncompetitive inhibitor of the V-nitrogenase-catalyzed reduction of all substrates except that of the proton (Dilworth et al., 1988) but, above 10% CO, it inhibits catalyzed proton reduction by 35% or more (Lee et al., 2009). Some, at least, of these electrons are diverted to catalyzed CO reduction to produce a variety of C_1-to-C_4 hydrocarbons (Hu et al., 2012; Lee et al., 2010). Since this discovery, exceedingly small quantities of similar CO reduction products have been detected with wild-type Mo-nitrogenase (Hu et al., 2011) and with somewhat higher yields from a variant Mo-nitrogenase (Yang et al., 2011). The physiological significance of this discovery is yet to be determined.

2.5 STRUCTURES OF THE NITROGENASE COMPONENT PROTEINS AND THEIR COMPLEXES

X-ray-based 3D structures of the wild-type Fe protein (Georgiadis et al., 1992; Jang et al., 2000; PDB code: 1FP6), the wild-type MoFe protein (Einsle et al., 2002; Howard and Rees, 1996; Kim and Rees, 1992a, b; Mayer et al., 1999; PDB codes: 1M1N, 1QGU, 1QH1, 1QH8, 3M1N), complexes of the two proteins (Chiu et al., 2001; Schindelin et al., 1997; Schmid et al., 2002a; Tezcan et al., 2005; PDB codes: 1N2C, 1G20, 1G21, 1M1Y 2AFH, 2AFI, 2AFK), and several variant Fe proteins (Chiu et al., 2001; PDB codes: 1G20, 1G21) and MoFe proteins (Mayer et al., 2002; Sarma et al., 2010; Schmid et al., 2002b; Sørlie et al., 2001; PDB code: 1FP4, 1H1L, 1L5H, 3K1A) are all known. There are no 3D structures of either the V-nitrogenase or the Fe-nitrogenase components or complexes. However, information from both Mössbauer (Krahn et al., 2002; Ravi et al., 1994) and X-ray absorption spectroscopies (Chen et al., 1993; George et al., 1988; Krahn et al., 2002) indicates that these nitrogenases share many structural similarities with the Mo-nitrogenase components (Smith et al., 2004).

When crystallized in the presence of excess MgADP, the Fe-protein structure shows one nucleotide bound to each subunit with both oriented parallel to one another in the subunit–subunit interface cleft (Jang et al., 2000). In the 2:1 Fe protein–MoFe protein complex, the nucleotides assume the same orientation and do not interact with the MoFe protein (see Fig. 2.2). Even though nucleotide binding to the Fe protein is known to be cooperative and to modify several properties of its $[Fe_4S_4]$ cluster, including its redox potential (Burgess and Lowe, 1996; Seefeldt and Dean, 1997), it remains unclear exactly how these effects are achieved. The nucleotides do not contact the $[Fe_4S_4]$ cluster directly, but rather their binding induces a significant structural change of the protein backbone in a region called switch II; this change is likely propagated to the cluster and so alters its electronic and redox properties (Seefeldt and Dean, 1997). A similar mechanism, called switch I, might be used for communication between the nucleotide-binding sites and the part of the Fe-protein surface that interacts with the MoFe protein during complex formation. Protein–protein contact could initiate electron transfer from the Fe protein and the resulting structural change in switch I would promote MgATP hydrolysis, followed by the dissociation of the Fe protein from the MoFe protein.

Early spectroscopic and controlled-degradation studies of the MoFe protein showed that it contained two previously unrecognized prosthetic groups, each of which contains about 50% of both the Fe and S^{2-} contents. They are the P cluster and the FeMo-cofactor (or M center). The composition and distribution within the protein of these metalloclusters was only established after the structure of the MoFe protein was solved. These unique prosthetic groups are distributed in pairs, one of each type, and one pair resides within each αβ-subunit pair. The P cluster has an $[Fe_8S_7]$ composition and is located at the interface of the α- and β-subunits, with each subunit providing three ligating cysteinyl residues. The FeMo-cofactor is an $[MoFe_7S_9(C^{4-})]$ cluster with an *R*-homocitrate molecule bound to the Mo atom. It is covalently bound to the α-subunit by only two

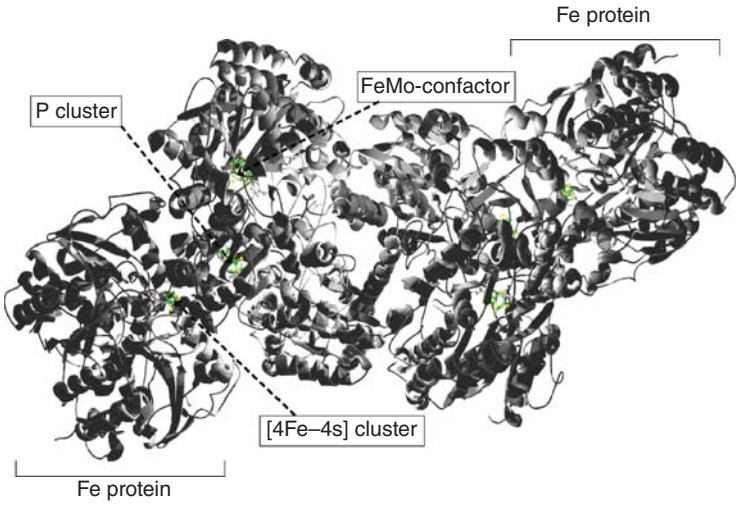

Figure 2.2 The structure of the 2:1 Fe protein–MoFe protein complex of the *Azotobacter vinelandii* nitrogenase stabilized by MgADP plus ALF_4^-. Both Fe-protein molecules bound to the centrally located MoFe protein are labeled and the three prosthetic groups are colored and labeled. Each FeMo-cofactor is situated within an α-subunit, whereas each P cluster sits at an interface of the α- and β-subunits. After docking, each Fe protein is located at an MoFe-protein α/β-subunit interface so that its [4Fe-4S] cluster is juxtaposed with a P cluster. The figure was drawn from coordinates available at PDB code: 1N2C.

amino acid residues (a cysteinyl and a histidinyl) and has no close involvement with the β-subunit (see Fig. 2.2).

A trapping technique, similar to that used for other "nucleotide switch" proteins, was successfully used with the Mo-nitrogenase components to produce the first stable complex of two Fe-protein molecules with one MoFe-protein molecule (Schindelin et al., 1997). This complex showed a significant conformational change in the Fe protein, but very little change in the MoFe protein. Each of the four Fe-protein subunits in the complex has an associated MgADP(AlF_4^-) moiety, which are again bound parallel in the subunit interface as found for the MgADP molecules bound to the Fe protein alone.

Docking of the two proteins occurs along their subunit interfaces and results in the Fe protein's [Fe_4S_4] cluster becoming buried in the protein–protein interface close to the MoFe protein's P cluster, which is then situated equidistant between the Fe protein's [Fe_4S_4] cluster and the FeMo-cofactor. This arrangement suggests a convenient electron-transfer pathway by which electrons move from the [Fe_4S_4] cluster through the P cluster to the FeMo-cofactor, where substrate reduction occurs. Another significant interaction involves the argininyl-100 residues of the Fe protein. In some bacteria, these residues are modified in a facile, reversible manner to regulate nitrogenase activity (Pope et al., 1985). The argininyl-100 residues have side chains that protrude into depressions in the surface of the MoFe protein and interact with the side chains of glutaminyl residues. Modification of these argininyl-100 residues introduces steric hindrance to the protein–protein interface, prevents complex formation, suppresses activity, and so regulates the enzyme.

Several other structures of 2:1 Fe protein–MoFe protein complexes have been solved, including one with a variant Fe protein (L127Δ-*Av*2) (Chiu et al., 2001), another from chemical cross-linking (Schmid et al., 2002a), and several with MgATP analogs (Tezcan et al., 2005). In all of these, only the Fe-protein structure is affected, the structure of the MoFe-protein component remains effectively unchanged. The last study clearly shows the importance of the nucleotide state of the Fe protein, that is, whether MgATP, MgADP, or neither is bound. The nucleotide state determines the docking geometry of the two proteins in the complex and may indicate that different circumstances induce different docking orientations or even sites. The docking option chosen, in turn, impacts the rate of electron transfer within the complex and might also direct the motion of one protein across the surface of the other.

2.6 STRUCTURES OF THE MoFe-PROTEIN PROSTHETIC GROUPS

The early reported structure of the FeMo-cofactor showed an [$MoFe_3S_3$] sub-cluster bridged by three μ_2-sulfides to an [Fe_4S_3] sub-cluster (see Fig. 2.3) (Chan et al., 1993; Kim and Rees, 1992b). As a result, only the terminal Fe atom of the latter sub-cluster had the expected tetrahedral geometry. All of the other six central Fe atoms had apparent trigonal geometry. However, a more recent, very high-resolution structure (Einsle et al., 2002) provided evidence for a single light atom, now generally considered to be carbide (C^{4-}) (Lancaster et al., 2011; Spatzel et al., 2011) within the central cavity of the FeMo-cofactor and equidistant from all six central Fe atoms (Fig. 2.3; see also Chapter 7). It is uncertain whether this light atom has a mechanistic or a structural role or both. The Mo atom has octahedral geometry provided by three sub-cluster μ_3-sulfides, the imidazole δ-N of an α-histidinyl residue, and by ligation from both the 2-hydroxyl and 2-carboxyl groups of *R*-homocitrate. The α-cysteinyl-Fe apex of the FeMo-cofactor has no associated water molecules, whereas the homocitrate-Mo apex is

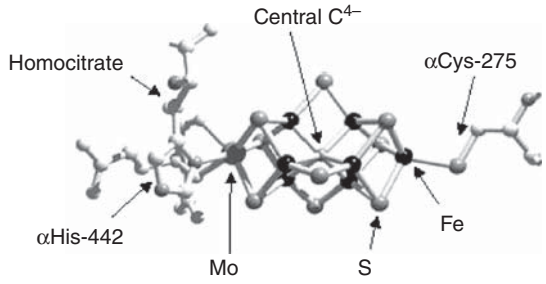

Figure 2.3 The structure of the FeMo-cofactor of *Azotobacter vinelandii* nitrogenase MoFe protein with its α-subunit-based ligating amino acid residues (αCys-275 and αHis-442) and homocitrate. The Mo, Fe, and S atoms are indicated as is the central carbide (C^{4-}). The seven darkest spheres are all Fe atoms and the eight lighter-gray spheres are all S atoms. The figure was drawn from coordinates available at PDB code: 1M1N.

surrounded by water molecules. Moreover, the homocitrate is positioned between the FeMo-cofactor and the P cluster and may be a component of an electron/proton-transfer pathway to bound substrate. There are also many important hydrogen-bonding interactions between the FeMo-cofactor and the surrounding amino acid residues (Kim and Rees, 1992a, b).

The P cluster is ligated by six cysteinyl residues, three from each subunit. The P cluster consists of an $[Fe_4S_4]$ sub-cluster that shares one of its sulfides with an $[Fe_4S_3]$ sub-cluster. This shared sulfide is in a very unusual situation as it is bound to all six of the central Fe atoms, that is, it is a μ_6-sulfide (see Fig. 2.4). The $[Fe_4S_4]$ sub-cluster is ligated by the γ-S of two α-subunit cysteinyl residues, whereas the $[Fe_4S_3]$ sub-cluster has ligation by the γ-S of two β-subunit cysteinyl residues. The two other cysteinyl residues, one from each of the α- and β-subunits, form μ_2-sulfide bridges between the sub-clusters.

When the P cluster in the protein is oxidized by redox-active dyes, it structurally rearranges to a more open structure with two of the four Fe atoms located in the $[Fe_4S_3]$ sub-cluster change ligation. They lose contact with the central μ_6-sulfide with one Fe atom becoming ligated by the γ-O of a β-serinyl residue and the other by the deprotonated backbone amide-N of the already bound and bridging α-cysteinyl residue (Fig. 2.4). Because both the serinyl – OH and the cysteinyl –NH are protonated when unbound and are deprotonated on binding, these redox-induced ligand changes raise the possibility that a two-electron oxidation of the P cluster during catalysis will also release two protons from the P cluster (Peters et al., 1997).

2.7 HOW ARE SUBSTRATES REDUCED?

Using the reductant sodium dithionite *in vitro*, the Fe protein is alternately oxidized and re-reduced as it delivers single electrons to the MoFe protein in a process that couples MgATP binding and hydrolysis to Fe protein–MoFe protein electron transfer and the association and dissociation of the two component proteins (see Fig. 2.5). The Fe protein alone is capable of binding MgATP, but both component proteins are required for MgATP hydrolysis. Neither component protein alone, with or without MgATP and/or reductant, will reduce substrate under the usual assay conditions (but see later). Use of a different reductant, for example, either Ti(III) or the *in vivo* reductant, flavodoxin hydroquinone, may impact one or more of the steps in this process (Duyvis et al., 1998; Erickson et al., 1999).

The overall reduction of N_2 to yield two molecules of NH_3 is thermodynamically favorable. So, why is MgATP required? If it is not a thermodynamic requirement, it

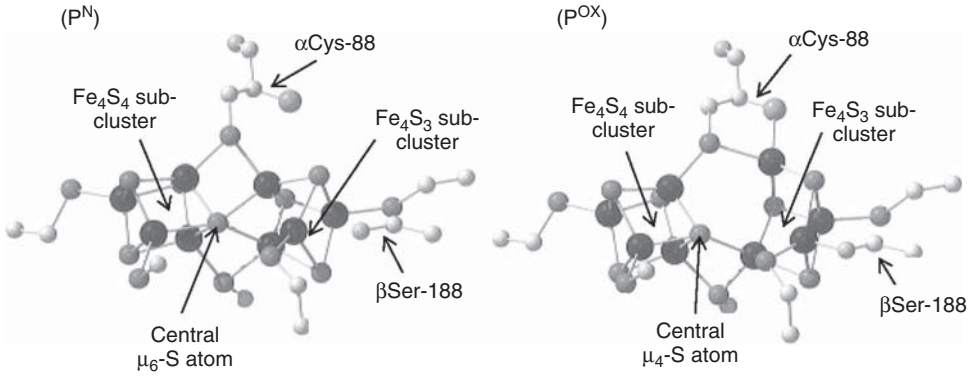

Figure 2.4 The structure of the P cluster of *Azotobacter vinelandii* nitrogenase MoFe protein in its dithionite-reduced (P^N) and oxidized (P^{OX}) states. Of its six ligating cysteinyl residues (αCys-62, αCys-88, αCys-154, βCys-70, βCys-95, βCys-153), only αCys-88 plus βSer-188 are identified because they are directly involved in the redox change. The Fe atoms are the larger darker spheres, the smaller lighter spheres are S atoms. *Source*: Modified from Peters et al., 1997 (PDB code: 3MIN and 2MIN).

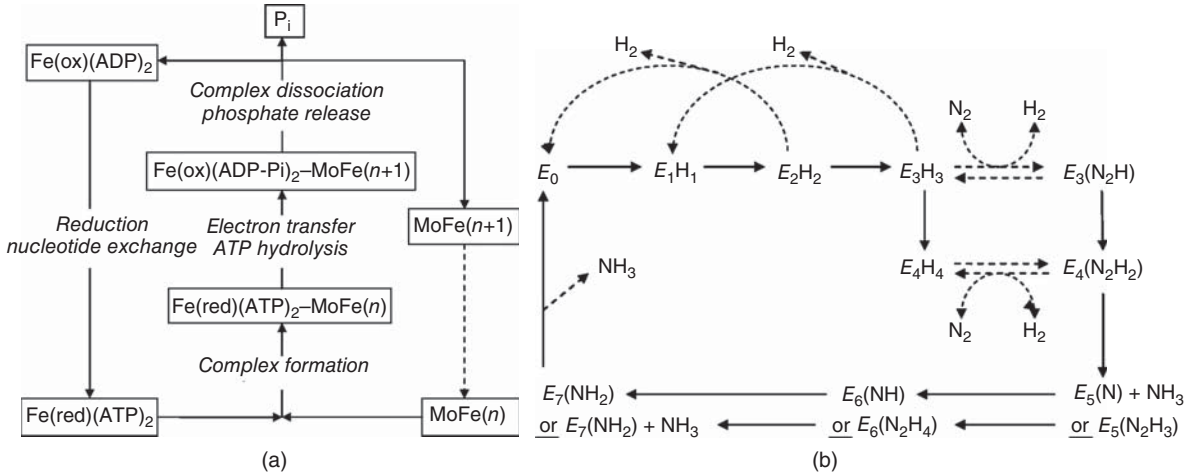

Figure 2.5 A modified Lowe–Thorneley scheme for Mo-nitrogenase catalysis. (a) The Fe-protein cycle describes the one-electron redox reactions of the Fe protein's [4Fe-4S] cluster, nucleotide exchange of the spent MgADP and phosphate (P_i) for MgATP, and complex formation with and electron transfer to the MoFe protein, when dithionite is used as reductant. Each turn of this cycle adds one electron to the MoFe protein. Fe represents the Fe protein in its oxidized (ox) or one-electron reduced (red) state; MoFe represents the MoFe protein with the number of electrons accepted shown as (n) or ($n+1$); the dashed arrow indicates that the MoFe($n+1$) protein can reenter this cycle and accept additional electrons and protons. *Source*: Modified from Lowe and Thorneley, 1984. (b) The MoFe-protein cycle for N_2 reduction. The dithionite-reduced (resting state) of the MoFe protein is designated as E_0 and, with each turn of the Fe-protein cycle, the MoFe protein goes through a succession of increasingly reduced states (E_1, E_2, … , E_7) as electrons and protons are accepted until sufficient and are accumulated for substrate reduction. An important concept of this scheme is that different substrates bind reversibly to different MoFe-protein redox states, for example, N_2 binds (as shown) at either E_3 or E_4 most likely by displacing H_2, whereas C_2H_2 binds at either E_1 or E_2. H_2 may be evolved from several redox states (as shown). The reduced-nitrogen intermediates shown are postulated and not proved (also see Fig. 2.6). Each solid arrow (⟶) represents one turn of the Fe-protein cycle, i.e., one hydrogenation (H^+/e^-) event with concomitant MgATP hydrolysis and each dashed arrow (- - -▶) represents a substrate-binding or product-release event. *Source*: Modified from Lowe and Thorneley, 1984.

must be for kinetic reasons. Most likely, MgATP binding helps drive electron transfer toward substrate reduction by increasing the difference in redox potential of the electron donor and acceptor (Lanzilotta and Seefeldt, 1997) and its subsequent hydrolysis, and complex dissociation ensures the irreversibility of the reaction. This so-called gating mechanism allows no backflow of electrons to the Fe protein and prevents energy-wasting futile cycling of electrons (Howard and Rees, 1996; Seefeldt and Dean, 1997). In this way, multiple electrons are accumulated within the MoFe protein and its bound substrate.

This view of the catalytic process actually describes only one part of the process, the so-called Fe-protein cycle. A more detailed description has been developed that involves two interconnecting processes: the Fe-protein cycle (Fig. 2.5a) and the MoFe-protein cycle (Fig. 2.5b) (Lowe and Thorneley, 1984). Here, a mechanistic simplification, which treats each αβ-subunit pair (with its encapsulated prosthetic groups) of the MoFe protein as an independently operating catalytic entity, is employed, even though long-range interactions between the two αβ-subunit pairs have been detected (Clarke et al., 2000; Maritano et al., 2001). The MoFe-protein cycle involves the progressive reduction of the MoFe protein (plus bound substrate) by up to eight electrons for N_2 binding and reduction, which therefore requires eight turns of the Fe-protein cycle. Partially reduced-nitrogen intermediates must remain on the enzyme until the reduction cycle is finished because NH_3 is the only product of catalyzed N_2 reduction by Mo-nitrogenase. Major concerns are how and where the eight electrons necessary for the reduction of each N_2 (accompanied by one H_2) are accommodated within the MoFe protein and how the required protons are delivered. This situation has been simplified by the assertion that the metal core of the FeMo-cofactor has only two accessible redox states, the EPR-active resting state (called M^N) and the one-electron-reduced EPR-silent state (called M^R) (Doan et al., 2011). All other electrons delivered from the Fe protein must, therefore, reside on either partially reduced substrate or inhibitor.

For N_2 to be bound to the active site, either three or four electrons must have been accumulated within the MoFe protein. If only one electron can be accommodated by the FeMo-cofactor, which would then become EPR-silent, where do the other two electrons reside before N_2 binds? Most likely, they reside as either a hydride (H^-) or hydrogen atom ($H^\bullet$) that subsequently becomes either H_2 or used to partially reduce N_2 (Dance, 2005, 2006). Similarly, the four-electron-reduced MoFe protein (labeled as E_4H_4 in Fig. 2.5) would equate to an oxidized EPR-active FeMo-cofactor with two bound hydrides. In fact, this

latter conclusion has been experimentally demonstrated with both hydrides proposed as μ^2-bridges between Fe atoms (as Fe – H – Fe) on the FeMo-cofactor rather than as either Mo – H – Fe or on μ^2-/μ^3-sulfur atoms (as > S – H) (Igarashi et al., 2005; Lukoyanov et al., 2010). Furthermore, the four-electron-reduced MoFe protein has been shown to relax to its resting state in a two-step process: the first step involves evolution of H_2 and formation of the two-electron-reduced MoFe protein (E_2H_2 in Fig. 2.5), which subsequently evolves a second H_2 to form the resting state (E_0 in Fig. 2.5) (Lukoyanov et al., 2007). Other substrates and inhibitors may, of course, be bound before three electrons have been accepted (Lowe and Thorneley, 1984; Lowe et al., 1993). For example, CO binds after only two electrons have arrived at the MoFe protein. Because the resulting complexes are EPR-active, both electrons must reside either on the bound CO (possibly as –C(H) = O) or as H^- (or $H^\bullet$).

As stated earlier, substrates and inhibitors only bind to Mo-nitrogenase under turnover conditions. The first insights into where and how these small molecules bind to the MoFe protein came from studies using the potent inhibitor CO. Because it is not reduced to any significant extent while wild-type Mo-nitrogenase is catalyzing the production of H_2 from protons, its bound forms should be present in relatively high concentration. During turnover under CO, the $S = 3/2$ EPR signal arising from the resting-state FeMo-cofactor within the MoFe protein disappears and one of two $S = 1/2$ EPR signals appear. A rhombic signal appears at low CO concentrations (p(CO) < 10 kPa), whereas an axial signal results at higher CO concentration (p(CO) > 0 kPa) (Cameron and Hales, 1998; Davis et al., 1979). Under either condition, only up to 50% of the total enzyme is present as the $S = 1/2$ EPR-active entity, the remainder is in the form of undetectable (EPR-silent) enzyme-based species, some (at least) of which will have CO bound. Using isotopically labeled materials at low p(CO), only one CO was found to be bound in the rhombic-EPR species called lo-CO, most likely as a bridge between two Fe atoms of the FeMo-cofactor. At the higher p(CO), two CO molecules were bound to produce the axial-EPR species called hi-CO; both CO molecules were suggested to be terminally bound, most likely to different Fe atoms (Lee et al., 1997). Furthermore, lo-CO and hi-CO could be interconverted by either increasing or decreasing the p(CO), which suggested that the single-bridging CO present in lo-CO converts to a terminal CO in hi-CO (Cameron and Hales, 1998; Lee et al., 1997; Maskos and Hales, 2003). A third minor CO-bound species, with an $S = 5/2$ EPR signal and called hi(5)-CO, was also detected under higher p(CO) and has been suggested to have two bridging CO molecules bound (Cameron and Hales, 1998).

An alternative approach for determining the binding modes of CO to wild-type Mo-nitrogenase involves the use of photolysis and an infrared detection system (Yan et al., 2011). This system is not limited to EPR-active species, the CO-binding mode can be determined for any photoactive entity by the measurement of its vibrational frequency. In fact, high concentrations of CO-bound products that do not correlate with any of the EPR-active species have been detected and analyzed (Yan et al., 2012). This approach has clearly shown that the bound CO in lo-CO retains its bridging mode even when a second terminal CO is bound to give hi-CO. Moreover, only the terminally bound CO can be photolyzed off of the hi-CO species. These results are consistent with stopped-flow FT-infrared studies of wild-type Mo-nitrogenase turning over under CO, where only one intense CO vibration (plus two considerably weaker) from a terminally bound CO molecule was produced under high p(CO), whereas under low p(CO), an initial terminal-CO vibration rapidly decayed to give a bridging CO mode (George et al., 1997; Thorneley et al., 2000). All these studies together suggest that this bridging CO bound under low p(CO) is the primary agent of inhibition of Mo-nitrogenase-catalyzed substrate reduction.

Although CO binds to V-nitrogenase, as shown by its effective reversible inhibition of V-nitrogenase-catalyzed substrate reduction (Dilworth et al., 1988), no EPR-active species have yet been observed for V-nitrogenase turning over under a CO atmosphere (Moore et al., 1994). Any complex that is formed must await future investigation.

The same EPR-based freeze-trapping technique has shown that C_2HR (where R = –H or $–CH_2OH$) and CN^- interact with the FeMo-cofactor, mostly clearly when the variant MoFe proteins, especially those with either single or double substitutions at the α-valinyl-70 and α-histinidyl-195 residues, are used (Dos Santos et al., 2005; Igarashi and Seefeldt, 2003). In an extension of this technique to the catalyzed N_2-reduction pathway, using labeled diazene, methyldiazene, or hydrazine (Hoffman et al., 2009), it was hoped that species that reflected early-, mid-, and late-stage intermediates would be trapped, but all three substrates produced the same two FeMo-factor-bound intermediates. Both of these trapped intermediates, described as M – NH_2 (for intermediate *H*) and M – NH_3 (for intermediate *I*), have a single nitrogen atom and so are produced after N–N bond cleavage (Lukoyanov et al., 2011; 2012). Because they are the expected end-products of almost any likely N_2-reduction pathway, they have little to add in this regard. Unfortunately, the chemical nature of the trapped intermediate after turnover under N_2 is the least clearly defined (Barney et al., 2005, 2009). Of course, the enzymes in these samples are not catalytically synchronized, and so only a portion of the population is trapped with other states lying undetected. Even so, the results of these and related relaxation studies have enabled links to be forged between the kinetically derived E_nH_n Lowe–Thorneley redox states of the MoFe protein and some spectroscopically characterized catalytic

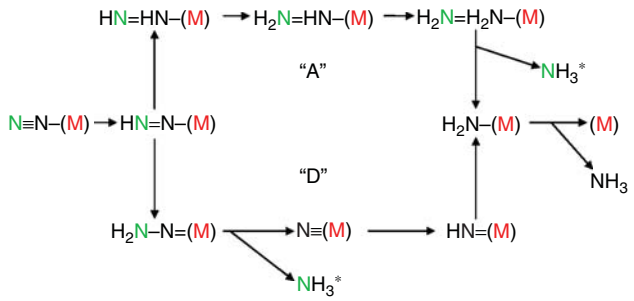

Figure 2.6 The Mo-nitrogenase N_2-reduction pathway alternatives. "A" represents the "alternating pathway," "D" the "distal pathway", (**M**) the M center (FeMo-cofactor) of the MoFe protein, **N** the distil nitrogen atom of the bound N_2 molecule, and each arrow a hydrogenation (H^+/e^-) event. A major consequence of the two different patterns of hydrogenation is that the first ammonia (**N**H_3*) is produced after only three hydrogenation events for the "D" pathway versus five for the "A" pathway. *Source*: Modified from Hoffman et al., 2009.

intermediates (Lukoyanov et al., 2007). Together with other considerations, these connections have led to the suggestion that the Mo-nitrogenase-catalyzed N_2-reduction pathway most likely occurs on Fe and via an "Alternating" hydrogenation pathway (Hoffman et al., 2009; Lukoyanov et al., 2012).

But what is an "Alternating" hydrogenation pathway? The Mo-nitrogenase-catalyzed N_2-reduction pathway can be constructed in two basic forms: both of which assume that N_2 is bound "end-on," that is, by only one (the proximal atom) of its two N atoms. One pathway, the so-called Distal pathway, has the distal (unbound) N atom being completely hydrogenated and released as NH_3 before the proximal N atom begins to be hydrogenated. The other pathway, the so-called Alternating pathway, has the two N atoms being alternately hydrogenated. These pathways are clearly delineated by the different intermediates predicted and the timing of product appearance (Fig. 2.6). Clear experimental evidence distinguishing between these two options remains elusive. Theoretical computations have been linked with both pathways; the "Distal" pathway is favored for catalysis at Mo, but the "Alternating" pathway is preferred if catalysis occurs at an Fe site.

2.8 WHERE ARE THE BINDING SITES FOR SUBSTRATES AND INHIBITORS?

It has long been assumed that binding and subsequent reduction of substrates occur at one or more metal atoms, but direct evidence has been hard to come by until recently (see earlier). Early circumstantial evidence for a role for the FeMo-cofactor in substrate binding came through variant nitrogenases (Burgess and Lowe, 1996; Scott et al., 1990; Smith et al., 2004). But where on FeMo-cofactor? The situation is complicated by a variety of observations that indicate the existence of more than one binding site for certain substrates and inhibitors. For example, two C_2H_2-binding sites have been detected by detailed kinetic analyses (Christiansen et al, 2000; Davis et al., 1979; Han and Newton, 2004; Shen et al., 1997), both of which can be occupied in the presence of CO and so are distinct from at least one of the two CO-binding sites (Shen et al., 1997). By the judicious use of combinations of substrates and inhibitors, a high-affinity ($K_m \sim 0.1\%$) C_2H_2-binding site, called site-2, has been implicated with N_2 binding and reduction and, therefore, is the physiologically relevant site (Han and Newton, 2004). Other studies with a variant enzyme indicated that the two C_2H_2-binding sites were close together and both were able to bind CO (Christiansen et al., 2000). If all this is so, then CO is likely reduced to various hydrocarbons by binding (at high p(CO)) to the physiologically relevant N_2-binding site-2, and the noncompetitive inhibitory effect of CO on substrate reduction would be the result of its binding (at low p(CO)) to the physiologically irrelevant site-1. These suggestions indicate that the responsible agent of substrate inhibition is the bridging CO bound as the lo-CO form of the enzyme. Furthermore, it suggests that the hi-CO form produced at high p(CO) reflects a catalytic intermediate in the pathway of CO reduction at site-2.

Which atoms of FeMo-cofactor bind these small molecules? Theoretical calculations are now coming to grips with the physical size, the chemical nature, the total-spin state, and the oxidation states of the constituent metal atoms of the FeMo-cofactor during catalysis (see, e.g., Harris and Szilagyi, 2011 and the references therein; see also Chapter 7). Early on, the preferred mode of N_2 binding was as a bridge between either two or four of its central Fe atoms, but these modes resulted in a small N_2-binding energy and, therefore, a weak bond. Also, immediate N_2 cleavage to metal nitrides was unfavored, which is consistent with (some) protonation of bound N_2 before bond cleavage. Now, since the discovery of the central light atom (initially assumed to be nitride, but almost certainly carbide), these various combinations of bridging Fe–N_2–Fe interactions at an Fe_4 face (Barney et al., 2006; Hoffman et al., 2009) appear to be very much less likely than binding at either a single Fe atom or an Fe_2 edge (Dance, 2006, 2011a, b). The interactions of substrates and inhibitors with the FeMo-cofactor described earlier are suggested to occur at one (the so-called Fe_6 atom; see Fig. 2.3) of the six centrally located Fe atoms with a sometime involvement of a second, the so-called Fe_2 atom (see, e.g., Dance, 2011a), although the Mo atom has had its proponents (Durrant, 2002, 2004). If all this is so, then Fe_6 of the FeMo-cofactor may be congruent with the physiologically relevant site-2 and Fe_2 with site-1.

2.9 HOW ARE ELECTRONS AND PROTONS DELIVERED?

The current dogma is that electrons are delivered by the Fe protein first to the P cluster, then through the protein matrix to the FeMo-cofactor, and finally to bound substrate. In support of this pathway, the structure of the Mo-nitrogenase complex shows the P cluster located midway between the [4Fe-4S] cluster of the Fe protein and the FeMo-cofactor. Although this arrangement could be coincidental, the redox-driven structural rearrangement of the P cluster (see earlier) supports an electron-transfer role (Peters et al., 1997). Support also comes from substituting the P-cluster-ligating β-cysteinyl-153 ligand with serinyl, which produces a variant MoFe protein that has a normal FeMo-cofactor, gives normal substrate-reduction products, and interacts usually with the Fe protein. It cannot, however, match the wild-type's maximum rate of substrate reduction. The most likely explanation is that intra-MoFe-protein electron transfer has been compromised (May et al., 1991). Furthermore, when the α-serinyl-188 residue (which binds to the P cluster when it is chemically oxidized) is substituted by cysteinyl, a new $S = 1/2$ EPR signal appears. This signal disappears when Fe protein, MgATP, and reductant are added (Chan et al., 1999). These observations indicate that the P cluster may be redox active during turnover.

In contrast, two early spectroscopic observations are inconsistent with this proposal. First, there is no change in the Mössbauer spectrum of the P clusters, which would indicate redox activity, during nitrogenase turnover (Smith et al., 1973). Second, electron transfer from the Fe protein results in the rapid loss of the FeMo-cofactor's $S = 3/2$ EPR signal, which suggests that electrons may go directly to this prosthetic group. Furthermore, a more recent freeze-trapping Mössbauer study indicates that the P clusters do not obviously change their redox state during turnover under low electron-flux conditions, even though the FeMo-cofactor accepts an electron (Fisher et al., 2007). So, under some conditions at least, electrons from the Fe protein may bypass the P cluster and go directly to the FeMo-cofactor and, if so, the P clusters might have an N_2 reduction-specific role in catalysis and, only at the highest electron-flux rates, provide the assistance necessary to commit bound N_2 to the irreversible reduction pathway (Lowe et al., 1993). However, a one-electron transfer from the Fe protein to the P cluster has been measured to be much faster than a one-electron transfer from the P cluster to the FeMo-cofactor (Danyal et al., 2011). In such circumstances, an oxidized P cluster would never be observed. But what if the P cluster undergoes a two-electron oxidation during turnover as suggested from structural studies (Peters et al., 1997)? Furthermore, is there only one or are there several electron-transfer pathways within the MoFe protein (Peters et al., 1995)? Is the P cluster involved in all of these pathways? Which substrates are serviced by which pathway?

Three likely proton-transfer routes have been identified (Durrant, 2001; Igarashi and Seefeldt, 2003). The first is a water-filled channel that stretches from the surface of the MoFe protein between the α- and β-subunits to the interstitial pool of water molecules around the homocitrate-Mo end of the FeMo-cofactor. This water-filled channel could rapidly deliver protons to bound substrate (Dance, 2005) and might also provide a pathway for substrates and products to diffuse to and from the reduction site(s). The arrival of electrons at the FeMo-cofactor increases the basicity of its sulfur atoms, which can then be protonated by water molecules in the interstitial pool. These bound protons may then be reduced to hydrogen atoms that can migrate to the Fe (or other) atoms to effect substrate reduction (Dance, 2006).

The other two potential pathways are proton-relay systems. The first involves the hydrogen bond between the imidazole of the side chain of α-histidinyl-195 and a central μ_2-sulfide (labeled S2), which bridges Fe_6 and Fe_2 of the FeMo-cofactor. This strictly conserved histidinyl residue is known to play an essential role in N_2 reduction (Dilworth et al., 1998). The same imidazole ring forms a hydrogen bond through an intervening water molecule to α-tyrosinyl-281, which is close to the protein's surface and flanked by two potential proton-capturing histidinyls, α-histidinyl-196 and α-histidinyl-383. This relay system could provide protons on demand. The second relay is more complex and involves three water molecules and three histidinyl residues, one of which (α-histidinyl-362) is on the surface and may capture protons for this relay. This path terminates in a different central bridging sulfide of FeMo-cofactor. These proton-relay systems would require a switch between alternative hydrogen-bonding networks as each proton is delivered and the system is realigned. These histidinyls are probably unable to compete with a water-filled channel to deliver multiple protons quickly enough, which suggests that the interstitial channel may be the primary route of proton delivery to substrates.

2.10 SOME CONCLUDING REMARKS

Although tremendous progress has been made, many questions remain concerning the mechanism of biological nitrogen fixation. The stringent requirements for N_2 binding and reduction appear to be satisfied by only a single site on the MoFe protein, whereas many of the alternative more easily reduced substrates and the inhibitor, carbon monoxide, have more than one binding and reduction site. Although sorting out their relevance to the overall functioning of the enzyme continues, there are several other mechanistic challenges that need answers. First, are both component proteins

and complex formation required for activity? Second, is MgATP hydrolysis absolutely required? Third, is the Fe protein the obligate electron donor?

For wild-type Mo-nitrogenase with sodium dithionite as the sole reductant *in vitro*, the answer to all three questions is "yes." However, if flavodoxin hydroquinone, which is the only physiological reductant in some organisms, is used *in vitro* (and maybe *in vivo*) to support substrate reduction, the MoFe protein/Fe protein complex does not dissociate during turnover (Duyvis et al., 1998). Some variant proteins go even further. The L127Δ Fe protein, for example, not only forms a stable complex with the wild-type MoFe protein but also completes intracomplex electron transfer without MgATP hydrolysis (Lanzilotta et al., 1996). A variant β-histidinyl-98 MoFe protein, when supplied with a low-potential Eu(II)-based reductant, catalyzes hydrazine reduction to ammonia without both the Fe protein and MgATP (Danyal et al., 2010).

An alternative approach has been to use photoreduction systems. An early success involved a light-driven process using eosin as the photo-sensitizer and NADH as the sacrificial electron donor, when Mo-nitrogenase-catalyzed photoreduction of both protons and acetylene was achieved (Druzhinin et al., 1993). A non-dissociating 1:1 MoFe protein/Fe protein complex was suggested to be the catalytic entity in a process that was more effective than with dithionite as electron donor, but still required MgATP. Photoreduction has since been taken one level further by using a surface-tethered ruthenium photo-sensitizer with dithionite as the sacrificial electron donor (Roth et al., 2010; Roth and Tezcan, 2012). In the absence of both Fe protein and MgATP, the modified MoFe-protein catalyzed proton, acetylene, and HCN photoreduction. These observations clearly indicate that neither the Fe protein nor MgATP is absolutely essential for Mo-nitrogenase-catalyzed substrate reduction. However, the challenge to develop a system that can be operated continuously or cyclically remains.

Mankind's current fixed-nitrogen requirements have outstripped the capacity of current biological nitrogen-fixing systems with the Haber–Bosch process supplying the balance. The final question is always whether biological nitrogen fixation can again become the major player in the delicate balance needed among environmental concerns and sufficient food production for the future (Dilworth et al., 2008; Leigh, 2004; Smil, 2001; Werner and Newton, 2005). In this context, we might ask why have some prokaryotes retained (or acquired) the alternative nitrogenases, especially when there are very few areas of the World where molybdenum concentrations are limiting. Do they serve some purpose other than nitrogen fixation? Will what we learn from these nitrogenases help in efforts to either enhance the nitrogen-fixation capabilities of microorganisms or endow new organisms and plants with this capability? Will they have a role in developing new commercial nitrogen-fertilizer production systems? We do not know; we will have to see what insightful creative research can produce.

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Chapter 3

Evolution and Taxonomy of Nitrogen-Fixing Organisms with Emphasis on Rhizobia

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3.1 INTRODUCTION

Biological nitrogen fixation (BNF) is a process by which atmospheric dinitrogen gas (N_2) is reduced to ammonia under natural conditions:

$$N_2 + 8[H] \Longleftrightarrow 2\,NH_3 + H_2$$

Contrary to the industrial reduction of N_2 by the Haber–Bosch process, which requires high temperature and pressure, BNF utilizes biologically stored solar energy to force the inert N_2 molecule to combine with hydrogen (H) and produce ammonia (NH_3) at ambient temperatures. N_2 is inert in relation to most biological systems, whereas ammonia can be used by living organisms as a nitrogen source or converted into nitrate (NO_3^-) for further use in the generation of proteins, nucleic acids, and other crucial compounds of the cells. Mineral nitrogen (NH_4^+, NO_3^-) fertilization of crop plants in agriculture forms the basis for modern agriculture, often at the expense of the utilization of BNF (Stoddard et al., 2009). However, it is estimated that 1 kg of industrially reduced nitrogen requires 1.5 kg of oil, BNF is not dependent on fossil fuels but uses energy derived from the sun. In agriculture, BNF by legumes in symbiosis with nitrogen-fixing bacteria can provide all the nitrogen necessary for plant growth under optimal growth conditions (Herridge et al., 2008). The solar energy used for BNF is bound in ATP and reducing power produced in the nitrogen-fixing organisms. In the case of symbiotic BNF with plants or photosynthetic microorganisms, the solar energy is captured by the photosynthetic system and stored in carbon compounds, which are transported to the site of nitrogen fixation, in the case of legumes to the root or stem nodules, in which nitrogen-fixing bacteria reside (Udvardi and Poole, 2013). These in turn use their metabolic machinery for the production of ATP and reducing power needed for BNF. In nonsymbiotic organisms, the energy is provided by corresponding energy-yielding mechanisms. It is estimated that the reduction of 1 mole of N_2 requires 16–24 moles of ATP (Franche et al., 2009). Thus, BNF is always an investment for plants and bacteria, both during the process and, for example, while legumes construct nodules to harbor the bacteria. In a recent review, Lindström and Mousavi (2010) summarize key features of BNF and its importance for the input of N to the biosphere and also give some recent figures of amounts of N fixed in diverse systems.

3.1.1 Evolution of Nitrogenase

The nitrogenase enzyme complex is the center for the BNF process. Nitrogenase itself will be dealt with in Chapters 2 and 7 in this book. From an evolutionary point of view, the origin of nitrogenase is still obscure. In present-day nitrogen fixers, a set of genes named *nif* (e.g., *nifH*, *nifD*, and *nifK* encoding the core subunits of nitrogenase) is present in most nitrogen-fixing organisms (for a recent review, see Franche

Biological Nitrogen Fixation, Volume 1, First Edition. Edited by Frans J. de Bruijn.

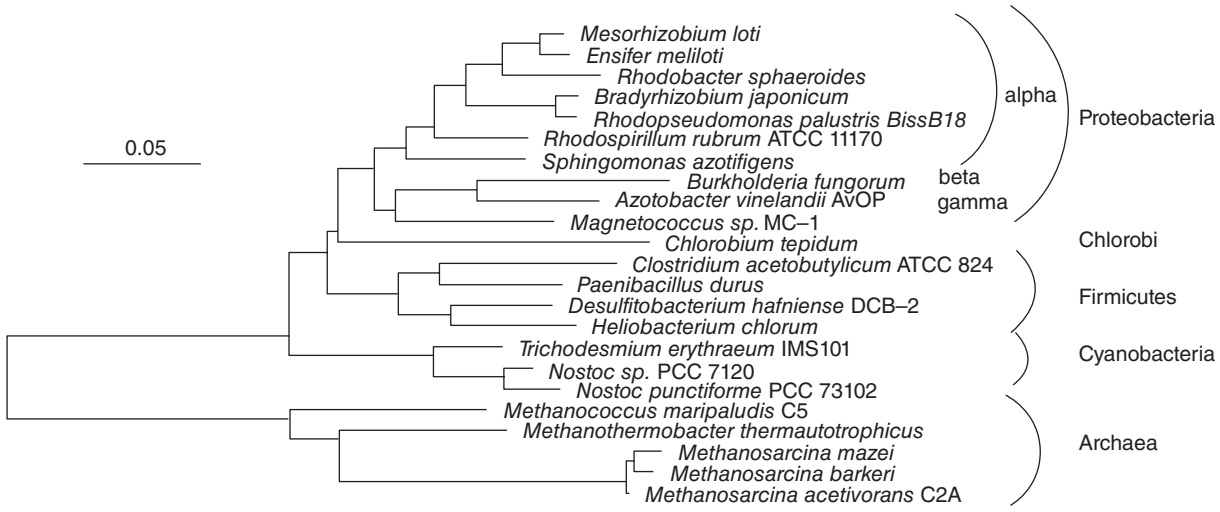

Figure 3.1 Phylogenetic 16S rRNA gene tree with prokaryotes carrying *nif* genes (by courtesy of German Jurgens). *Source*: Reprinted from Franche et al. (2009) with the kind permission of Springer-Verlag.

et al. (2009)). The genes *nifHDK* can be used as a genetic marker when studying the occurrence of nitrogen-fixing organisms in diverse taxa (Fig. 3.1).

An important component of nitrogenase enzyme complexes is the metal cofactor. The genes *nifHDK* encode a nitrogenase with an iron-molybdenum cofactor (FeMo-co). However, some nitrogen fixers use vanadium (V, genes *vnfHDK*) instead of Mo in the cofactor and some just iron (genes *anfHDK*) (Schüddekopf et al., 1993: see Chapter 2). It is a widespread perception that V- and Fe-nitrogenases are ancient forms of Mo-nitrogenase. Boyd et al. (2011) in their study of the evolution of nitrogenases came to a different conclusion. By examining multiple nitrogenase proteins phylogenetically and structurally, they proposed that Mo-nitrogenase first emerged within the hydrogenotrophic methanogenic archaea already before the "great oxidation event" (GOE), which occurred 2.45–2.22 billion years ago (Anbar et al., 2007). The alternative V- and Fe-nitrogenases then evolved in response to Mo limitation, and Mo-nitrogenase became prevalent when bioavailable Mo increased in the biosphere at the time of the GOE. According to the analyses by Fani et al. (2000), *nifDK* and *nifEN* (involved in the biosynthesis of the FeMo-cofactor) constitute a paralogous gene family that arose via gene duplications, perhaps even before the appearance of the last common ancestor (LUCA). They further propose that the *nifDKEN* genes were present before the divergence of *Bacteria* and *Archaea.*

This suggestion was supported by results compiled in a mini-review by Zehr et al. (2003), who most comprehensively describe how widespread nitrogenase (represented by *nifH*) is diverse across ecosystems and prokaryotic taxa. Hartmann and Barnum (2010) inferred phylogenies of 60 *nifK* and *nifDK* sequences across diverse prokaryotic phyla (represented by 16S rRNA gene sequence) and proposed that both horizontal and vertical gene transfers could explain the observed patterns though within phyla vertical transmission seemed to be prevalent (see also Chapters 16–19).

3.1.2 Evolution of Symbiotic Nitrogen Fixation

The evolution of symbiotic nitrogen fixation is in comparison with the evolution of *nif* genes, which is a recent phenomenon. By symbiosis we mean a situation where nitrogen-fixing bacteria enter a host plant and fix nitrogen inside the plant, most often to the benefit of both partners (Lindström and Mousavi, 2010). The symbiosis between leguminous plants and alpha- or betaproteobacterial species (rhizobia) is the best known and also agronomically most important. The symbiosis between bacteria from the genus *Frankia* with alders (*Alnus*) and other actinorhizal plants is widespread in nature (Franche et al., 2009; see Chapters 35, 42, 43, 48, 55), whereas symbioses between cyanobacteria and plants from the diverse genera *Azolla*, *Cycas*, and *Gunnera* are less known (Osborne and Bergman, 2009). Rhizobia invade their host plants via root hair or crack entry infection to enter the root nodule, which the host is constructing for the potentially beneficial microsymbionts (Sprent, 2001). Recently, similarities between arbuscular mycorrhizal and rhizobial infections have led to the proposal that nitrogen-fixing root symbioses have the same evolutionary origin as mycorrhizal ones. These aspects have recently been reviewed in terms of both signaling factors (Cough and Cullimore, 2011; see also Chapter 51) and central signaling pathways in the plant (Singh and Parniske, 2012). Figure 3.2 shows a range of evolutionary events regarding symbioses. Mycorrhizal symbioses are thought to predate the rhizobial

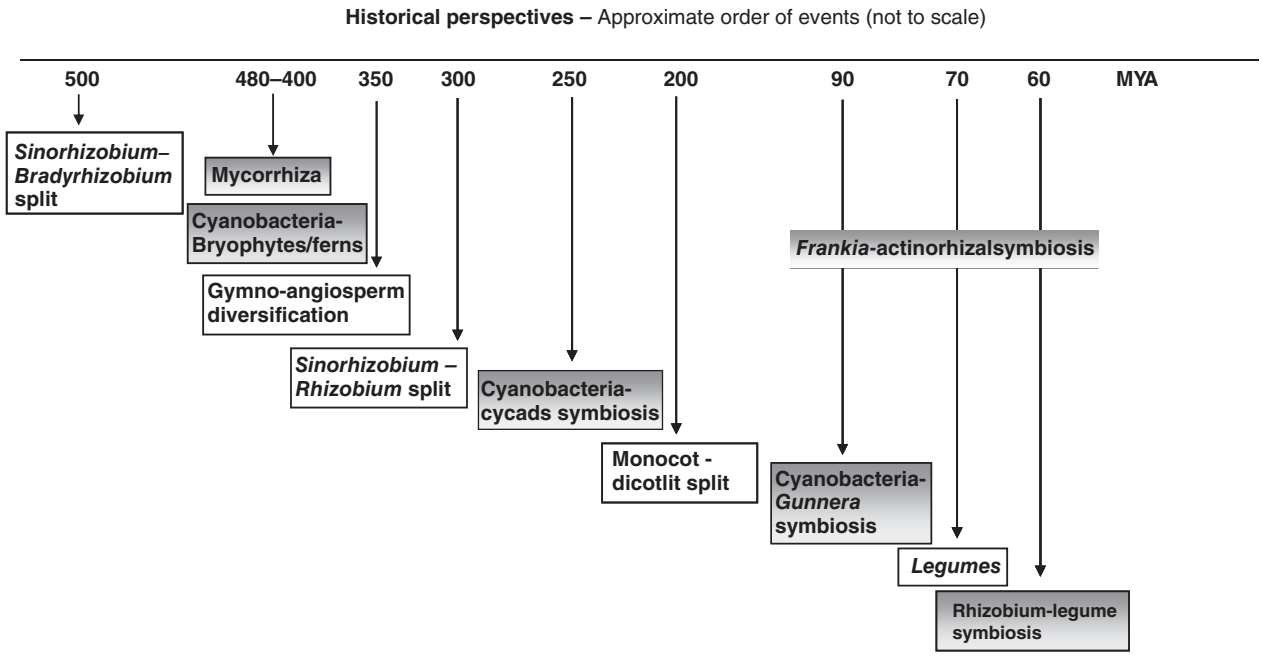

Figure 3.2 Approximate order of historical events important for the evolution of nitrogen-fixing symbioses, compiled from the sources Kistner and Parniske (2002), Lavin et al. (2005), Osborne and Bergman (2009), Sprent (2008), Turner and Young (2000), and Wang et al. (2010) and the references in them. *Source*: Reprinted from Lindström and Mousavi (2010) with the kind permission of John Wiley & Sons Ltd.

infections, which imply that the rhizobia adopted pathways in the plants already evolved in response to mycorrhizal infections.

Rhizobial symbionts are easy to cultivate in the laboratory even though most species do not fix N *ex planta* but use combined nitrogen. Thus, genes encoding both nitrogen fixation (*nif*, *fix*) and infection and nodulation (*nod*, *nol*, *noe*) have been studied since the 1980s. Forward and reverse genetic approaches have been used to assign gene functions, of which the activation of *nod* genes by plant root exudates and the expression of *nod* genes to produce lipo-chitooligosaccharide (LCO) signal molecules have been the main breakthroughs (for a recent review of the diversity of symbiotic interactions, see Masson-Boivin et al., 2010; see also Chapters 50 and 51). In legume–*Rhizobium* interactions, there is host specificity in terms of both nodulation and N fixation, the former being determined by the nature of *nod* gene inducing root exudate as well as the nature of the LCO signal molecules (Masson-Boivin et al., 2010). The specific roles of different *nod* gene products in the biosynthesis of LCOs are fairly well known, whereas the specificity of N fixation (effective vs ineffective symbiosis) is less clear cut.

The evolution of the *Rhizobium*–legume symbiosis has been proposed to be an example of co-evolution. However, there is so far no solid evidence that this is the case. True coevolution means that both partners undergo evolutionary changes to reach a common goal, the symbiosis, whereas so far only *Rhizobium* evolution under host plant constraint has been conclusively demonstrated (Suominen et al., 2001). Species producing LCO molecules with polyunsaturated fatty acid residues infect plants belonging to the same IRLC legume clade (Dresler-Nurmi et al., 2007), which is further proof for adaptation by rhizobia to legumes. Österman et al. (2011) found no evidence for adaptive evolution of symbiotic plant genes (NORK and *Nfr5*) in *Galega* species though there was diversity in the genes in the studied plant populations.

The adaptation of *Rhizobium* bacteria to their hosts is mediated by horizontal gene transfer (HGT). For a long time, it was already known that rhizobial symbiotic genes often reside on plasmids (Nuti et al., 1979, Hirsch et al., 1980). Freiberg et al. (1997) demonstrated by sequencing of a 500-kb plasmid (pNGR234a) from *Sinorhizobium* sp. strain NGR 234 that all functions needed for symbiosis as well as conjugal transfer were located on this plasmid (see also Chapter 32). Sullivan and Ronson (1998) described how a 500-bp gene region in *Mesorhizobium loti* strain ICMP3153 carrying genes encoding nodulation, nitrogen fixation, and vitamin biosynthesis was transferable to three other nonsymbiotic mesorhizobial genospecies, confirming a hypothesis arisen from field observations. This element was named the symbiotic island. It was found to integrate into mesorhizobial chromosomes by a phage-encoded integrase into a Phe-tRNA gene (see Chapter 21). Genomic (symbiotic) islands have later been found in other *Rhizobium* species, for example, *Bradyrhizobium japonicum* (Kaneko et al., 2002). Young et al. (2006) demonstrated that the genome structure of *Rhizobium leguminosarum*

strain Rlv3841 displayed both physically and functionally distinct compartments, which they named the core and the accessory genome. The accessory genes are those encoding niche adaptation, such as symbiotic host range. As they often reside on transmissible elements, they are prone to HGT. The core genes mainly undergo vertical transfer and encode housekeeping properties. The accessory genes are dispensable, whereas the core genes are indispensable for the bacteria. Harrison et al. (2010) further proposed that genetic elements that have both accessory and core properties should be named chromids. Chromids are, for example, rhizobial megaplasmids with both symbiotic and housekeeping functions (see also Chapters 25–27).

Rhizobial genomes are dynamic, which was a prerequisite for the evolution of extant symbiotic interactions. The *nif* genes at a higher taxonomic rank mainly seem to be vertically transferred within phyla, whereas rhizobial *nod* and *nif* genes show both vertical and horizontal transfer patterns (Haukka et al., 1998; Menna and Hungria, 2011; Aserse et al., 2012a).

HGT of symbiotic islands or plasmids are major genetic events that greatly change the phenotypic properties of the bacteria. Not only nodulation and nitrogen fixation but also other properties contributing to ecological niche adaptation can be genetically labile. Accessory genes and properties they encode are thus unsuitable for taxonomic classification of rhizobia, since loss of a symbiotic plasmid will, for example, greatly change the phenotype.

3.1.3 Taxonomy of Bacteria

Current taxonomic practice regarding bacteria requires that all validly described species can be distinguished from their nearest neighbors by at least one phenotypic feature (Tindall et al., 2010). The sequencing of 16S rRNA genes (but no other genes) and their phylogenetic comparison with close relatives is now obligatory for species descriptions and in most cases DNA:DNA hybridization should be used to delineate members of novel species from known ones (Roselló-Móra, 2011). The 16S rRNA gene is especially less variable in many rhizobial taxa than other housekeeping genes, and therefore its use might not properly delineate putative new species (Aserse et al., 2012a, 2012b). Unfortunately, DNA:DNA hybridization has become more like engineering than biological science, meaning that results are interpreted by rigidly applying percentage rules (70% relative homology is used as the borderline between species; (Tindall et al., 2010)) and forgetting uncertainty measures and variation between laboratories and methods.

According to a recent paper by Tamames and Roselló-Móra (2012), more and more novel species are described based on single isolates. This trend has led to a decrease in the impact factor for the *International Journal of Systematic and Evolutionary Microbiology (IJSEM)*, in which all new species should be validly described or listed. Despite the name of the journal, the description of new species is deviating from biology in a regrettable way. On the other hand, "invalidly described species" are increasing in amount, since partial 16S rRNA gene sequences are given names or called species without the corresponding culture being available, which is a prerequisite for a validly described species (Garrity and Oren, 2013).

The rules for naming prokaryotic species are published in the Bacteriological Code, published by the International Committee for the Systematics of Prokaryotes (ICSP) (Lapage et al., 1992). The rules for how to delineate species arise from the scientific community and are ultimately dependent on the *IJSEM* reviewers' opinions. To streamline procedures, the ICSP has subcommittees devoted to various taxa to assist in the taxonomic work. The ICSP Subcommittee on the Taxonomy of *Agrobacterium* and *Rhizobium* is responsible for developing guidelines for species descriptions among those genera.

Nowadays, the subcommittee has taken an interest in all taxa containing nodulating bacteria and also non-nodulating taxa related to the nodulating taxa. The subcommittee meets regularly and reports in the form of minutes from the meetings (recent minutes: Lindström and Young, 2009, 2011). The subcommittee has a website with useful information for rhizobial taxonomists and everybody interested in systematics of these interesting organisms. The website is maintained by Vinuesa (2013).

This subcommittee tries to operate on the borderline of taxonomy and biology. Thus, we consider the biology of the organisms as the baseline for taxonomy. Instead of dividing rhizobia based on single strains described by rigid but sometimes meaningless criteria, we advocate the use of multiple strains (to cover diversity) and an evolutionary or cross-disciplinary approach to speciation and taxonomy (Lindström and Gyllenberg, 2006).

The subcommittee is not consulted when new species are proposed. If this were the case, some mistakes could perhaps be avoided. Now phylogenetically close species have been given different names (e.g., *Rhizobium fabae* and *Rhizobium pisi*, some species consist of just one strain (e.g., *R. pisi*) and species are misplaced in new phylogenetic reconstructions (e.g., *Rhizobium indigoferae*) (Aserse et al., 2012b). The new species descriptions are often just snapshots of a small part of the phylogenetic tree and the diversity is hidden. We therefore decided to present phylogenetic overviews of currently described species of rhizobia and some related taxa.

3.2 MATERIALS AND METHODS

3.2.1 Analysis of 16S rRNA Genes

The sequences (>1300 bp) of 16S rRNA gene of 160 proteobacterial species were retrieved from GenBank

Table 3.1 List of strains representing the species used in figure 3.3 and the corresponding accession numbers

Number	Species	Accession Number
1	*Agrobacterium tumefaciens* ATCC 23308^T	D14500
2	*Agrobacterium fabrum* C58	NR_074266
3	*Agrobacterium ferrugineum* ATCC 25652^T	D88522
4	*Agrobacterium larrymoorei* AF3.10^T	NR_026519
5	*Agrobacterium meteori* NBRC 15793^T	AB680965
6	*Agrobacterium radiobacter* LMG 140^T	AM181758
7	*Agrobacterium rubi* LMG 17935^T	AM181759
8	*Agrobacterium vitis* NCPPB 3554^T	D14502
9	*Allorhizobium undicola* ORS 992^T	NR_026463
10	*Aminobacter anthyllidis* STM4645^T	FR869633
11	*Azorhizobium caulinodans* ORS 571^T	AP009384
12	*Azorhizobium doebereinerae* BR5401^T	NR_041839
13	*Azorhizobium oxalatiphilum* DSM 18749^T	FR799325
14	*Bosea lathyri* LMG 26379^T	FR774993
15	*Bosea lupini* LMG 26383^T	FR774992
16	*Bosea robiniae* LMG 26381^T	FR774994
17	*Bradyrhizobium arachidis* CCBAU 051107^T	HM107167
18	*Bradyrhizobium betae* PL7HG1^T	AY372184
19	*Bradyrhizobium canariense* BTA-1^T	NR_042177
20	*Bradyrhizobium cytisi* CTAW11^T	EU561065
21	*Bradyrhizobium daqingense* CCBAU 15774^T	HQ231274
22	*Bradyrhizobium denitrificans* IFAM 1005^T	NR_041827
23	*Bradyrhizobium elkanii* USDA 76^T	U35000
24	*Bradyrhizobium huanghuaihaiense* CCBAU 23303^T	HQ231463
25	*Bradyrhizobium iriomotense* NBRC 102520^T	AB681854
26	*Bradyrhizobium japonicum* USDA 6^T	AB231927
27	*Bradyrhizobium jicamae* PAC68^T	AY624134
28	*Bradyrhizobium lablabi* CCBAU 23086^T	GU433448
29	*Bradyrhizobium liaoningense* 2281^T	NR_041785
30	*Bradyrhizobium pachyrhizi* PAC48^T	AY624135
31	*Bradyrhizobium rifense* CTAW71^T	EU561074
32	*Bradyrhizobium yuanmingense* CCBAU 10071^T	AF193818
33	*Burkholderia caribensis* MWAP64^T	Y17009
34	*Burkholderia contaminans* LMG 23361^T	JX986975
35	*Burkholderia diazotrophica* NKMU-JPY461^T	HM366717
36	*Burkholderia fungorum* LMG 16225^T	AF215705
37	*Burkholderia lata* 383^T	CP000150
38	*Burkholderia mimosarum* PAS44^T	AY752958
39	*Burkholderia nodosa* Br3437^T	AY773189
40	*Burkholderia phymatum* STM815^T	AJ302312
41	*Burkholderia sabiae* Br3407^T	AY773186
42	*Burkholderia symbiotica* NKMU-JPY345^T	HM357233
43	*Burkholderia tuberum* STM678^T	AJ302311
44	*Cupriavidus necator* LMG 8453^T	AF191737
45	*Cupriavidus taiwanensis* LMG 19424^T	AF300324
46	*Devosia neptuniae* J1^T	NR_028838
47	*Herbaspirillum lusitanum* P6-12^T	AF543312
48	*Mesorhizobium mediterraneum* LMG 17148^T	NR_042483
49	*Mesorhizobium abyssinicae* AC98c^T	GQ847896
50	*Mesorhizobium albiziae* CCBAU61158^T	NR_043549
51	*Mesorhizobium alhagi* CCNWXJ12-2^T	EU169578
52	*Mesorhizobium amorphae* ACCC19665^T	NR_024879
53	*Mesorhizobium australicum* WSM2073^T	AY601516
54	*Mesorhizobium camelthorni* CCNWXJ40-4^T	EU169581

(*continued*)

Table 3.1 (*Continued*)

Number	Species	Accession Number
55	*Mesorhizobium caraganae* CCBAU 11299^T	NR_044118
56	*Mesorhizobium chacoense* PR5^T	NR_025411
57	*Mesorhizobium ciceri* NBRC 100389^T	AB681164
58	*Mesorhizobium gobiense* CCBAU 83330^T	EF035064
59	*Mesorhizobium hawassense* AC99b^T	GQ847899
60	*Mesorhizobium huakuii* NBRC 15243^T	AB680816
61	*Mesorhizobium loti* NZP 2213^T	NR_025837
62	*Mesorhizobium metallidurans* STM 2683^T	AM930381
63	*Mesorhizobium muleiense* CCBAU 83963^T	HQ316710
64	*Mesorhizobium opportunistum* WSM2075^T	AY601515
65	*Mesorhizobium plurifarium* LMG 11892^T	Y14158
66	*Mesorhizobium qingshengii* CCBAU 33460^T	JQ339788
67	*Mesorhizobium robiniae* CCNWYC 115^T	EU849582
68	*Mesorhizobium sangaii* SCAU7^T	EU514525
69	*Mesorhizobium septentrionale* SDW 014^T	AF508207
70	*Mesorhizobium shangrilense* CCBAU 65327^T	EU074203
71	*Mesorhizobium shonense* AC39a^T	GQ847890
72	*Mesorhizobium silamurunense* CCBAU 01550^T	EU399698
73	*Mesorhizobium tamadayense* Ala-3^T	AM491621
74	*Mesorhizobium tarimense* CCBAU 83306^T	EF035058
75	*Mesorhizobium temperatum* SDW018^T	AF508208
76	*Mesorhizobium thiogangeticum* SJT^T	AJ864462
77	*Mesorhizobium tianshanense* A-1BS^T	NR_024880
78	*Methylobacterium nodulans* ORS 2060^T	AF220763
79	*Microvirga lotononidis* WSM3557^T	HM362432
80	*Microvirga lupini* Lut6^T	EF191408
81	*Microvirga zambiensis* WSM3693^T	HM362433
82	*Ochrobactrum lupini* LUP21^T	NR_042911
83	*Ochrobactrum cytisi* ESC1^T	AY776289
84	*Phyllobacterium trifolii* PETP02^T	AY786080
85	*Rhizobium alkalisoli* CCBAU 01393^T	EU074168
86	*Rhizobium lupini* DSM 30140^T	NR_044869
87	*Rhizobium aggregatum* DSM 1111^T	X73041
88	*Rhizobium alamii* GBV016^T	AM931436
89	*Rhizobium borbori* DN316^T	EF125187
90	*Rhizobium cauense* CCBAU 101002^T	JQ308326
91	*Rhizobium cellulosilyticum* ALA10B2^T	NR_043985
92	*Rhizobium daejeonense* NBRC 102495^T	AB681832
93	*Rhizobium endophyticum* CCGE 2052^T	EU867317
94	*Rhizobium etli* CFN 42^T	NR_074499
95	*Rhizobium fabae* CCBAU 33202^T	DQ835306
96	*Rhizobium galegae* HAMBI 540^T	AB680726
97	*Rhizobium gallicum* R602^T	EU488748
98	*Rhizobium giardinii* H152^T	EU488750
99	*Rhizobium grahamii* CCGE 502^T	JF424608
100	*Rhizobium hainanense* NBRC 107132^T	AB682466
101	*Rhizobium halophytocola* YC6881^T	GU322905
102	*Rhizobium helanshanense* CCNWQTX14^T	GU201840
103	*Rhizobium herbae* CCBAU 83011^T	GU565534
104	*Rhizobium huautlense* S02^T	NR_024863
105	*Rhizobium indigoferae* CCBAU 71042^T	NR_025157
106	*Rhizobium trifolii* ATCC 14480	AY509900
107	*Rhizobium leguminosarum* USDA 2370^T	U29386

(*continued*)

Table 3.1 (*Continued*)

Number	Species	Accession Number
108	*Rhizobium leucaenae* CFN 299^T	X67234
109	*Rhizobium loessense* CCBAU 7190B^T	NR_028819
110	*Rhizobium lusitanum* P1-7^T	NR_043150
111	*Rhizobium mesoamericanum* CCGE 501^T	JF424606
112	*Rhizobium mesosinicum* CCBAU 25010^T	NR_043548
113	*Rhizobium miluonense* CCBAU 41251^T	NR_044063
114	*Rhizobium mongolense* USDA 1844^T	U89817
115	*Rhizobium multihospitium* CCBAU 83401^T	EF035074
116	*Rhizobium naphthalenivorans* TSY03b^T	AB663504
117	*Rhizobium nepotum* 39/7^T	FR870231
118	*Rhizobium oryzae* Alt 505^T	NR_044393
119	*Rhizobium petrolearium* SL-1^T	EU556969
120	*Rhizobium phaseoli* ATCC 14482^T	NR_044112
121	*Rhizobium phenanthrenilyticum* F11^T	FJ743436
122	*Rhizobium pisi* DSM 301232^T	AY509899
123	*Rhizobium pseudoryzae* J3-A127^T	DQ454123
124	*Rhizobium pusense* NRCPB10^T	FJ969841
125	*Rhizobium qilianshanense* CCNWQLS01^T	JX117881
126	*Rhizobium rhizogenes* ATCC 11325^T	AY945955
127	*Rhizobium rosettiformans* w3^T	EU781656
128	*Rhizobium selenitireducens* B1^T	NR_044216
129	*Rhizobium skierniewicense* Ch11^T	HQ823551
130	*Rhizobium soli* DS-42^T	EF363715
131	*Rhizobium sphaerophysae* CCNWGS0238^T	FJ154088
132	*Rhizobium subbaraonis* JC85^T	FR714938
133	*Rhizobium sullae* IS123^T	Y10170
134	*Rhizobium taibaishanense* CCNWSX 0483^T	HM776997
135	*Rhizobium tarimense* PL-41^T	HM371420
136	*Rhizobium tibeticum* CCBAU 85039^T	EU256404
137	*Rhizobium tropici* CIAT 899^T	CP004015
138	*Rhizobium tubonense* CCBAU 85046^T	EU256434
139	*Rhizobium vallis* CCBAU 65647^T	FJ839677
140	*Rhizobium vignae* CCBAU 05176^T	GU128881
141	*Rhizobium yanglingense* SH 22623^T	NR_028663
142	*Ruegeria atlantica* IAM 14463^T	NR_043449
143	*Shinella kummerowiae* CCBAU 25048^T	EF070131
144	*Sinorhizobium adhaerens* LMG 20216^T	AM181733
145	*Sinorhizobium americanum* CFNEI 156^T	AF506513
146	*Sinorhizobium arboris* HAMBI 1552^T	Z78204
147	*Sinorhizobium fredii* LMG 6217^T	X67231
148	*Sinorhizobium garamanticus* ORS 1400^T	AY500255
149	*Sinorhizobium kostiense* LMG 19227^T	AM181748
150	*Sinorhizobium kummerowiae* CCBAU71714^T	AF364067
151	*Sinorhizobium medicae* LMG 19920^T	L39882
152	*Sinorhizobium meliloti* LMG 6133^T	X67222
153	*Sinorhizobium mexicanum* ITTG R7^T	DQ411930
154	*Sinorhizobium numidicus* ORS 1407^T	AY500254
155	*Sinorhizobium saheli* LMG 7837^T	X68390
156	*Sinorhizobium sojae* CCBAU 05684^T	GU593061
157	*Sinorhizobium terangae* LMG 7834^T	X68388
158	*Sinorhizobium xinjiangensis* CCBAU 110^T	AF250354
159	*Stappia stellulata* IAM 12621^T	NR_043448
160	*Thalassobius gelatinovorus* IAM 12617^T	NR_043447

Table 3.2 List of Strains, Host Plants, and Accession Numbers of the Gene Sequences Included in Figures 3.4 and 3.5

Organism	Host Plant/ Isolation Source	*recA*	*glnII*	*atpD*	*gyrB*	*rpoB*	*dnaK*
B. rifense CTAW71^T	*Cytisus triflorus*	GU001585	GU001604				
B. huanghuaihaiense CCBAU 23303^T	*Glycine max*	HQ231595	HQ231639	HQ231682	JX437672	JX437679	JX437665
B. arachidis CCBAU 051107^T	*Arachis hypogaea*	HM107233	HM107251	HM107217	JX437675	JX437682	JX437668
B. canariense BTA-1^T	Genistoid legumes	AY591553	AY386765	AY386739	FM253220	FM253263	FM253306
B. denitrificans LMG 8443^T	Surface water*	EU665419	HM047121	FM253153	FM253239	FM253282	FM253325
B. elkanii USDA 76^T	*Glycine max*	AY591568	AY599117	AM418752	AM418800	AM295348	AJ431152
B. iriomotense EK05^T	*Entada koshunensis*	AB300996	AB300995	AB300994	HQ873308	HQ587646	JF308944
B. jicamae PAC68^T	*Pachyrhizus erosus*	HM047133	FJ428204	FJ428211	HQ873309	HQ587647	JF308945
B. lablabi CCBAU 23086^T	*Lablab purpureus*	GU433522	GU433498	GU433473	JX437670	JX437677	JX437663
B. liaoningense LMG 18230^T	*Glycine max*	AY591564	AY386775	AY386752	FM253223	FM253266	FM253309
B. pachyrhizi PAC48^T	*Pachyrhizus erosus*	HM590777	FJ428201	FJ428208	HQ873310	HQ587648	JF308946
B. yuanmingense CCBAU 10071^T	*Lespedeza* spp	AY591566	AY386780	AY386760	FM253226	FM253269	FM253312
B. japonicum USDA 6^T	*Glycine max*	AM182158	AF169582	AM168320	AM418801	AM295349	AM182120
B. betae LMG 21987^T	*Beta vulgaris* tumour*	FJ970378	AB353733	FM253129	FM253217	FM253260	FM253303
B. dagingense CCBAU 15774^T	*Lablab purpureus*	HQ231270	HQ231301	HQ231289	JX437669	JX437676	JX437662
B. cytisi CTAW11^T	*Cytisus villosus*	GU001575	GU001594	GU001613	JN186292	JN186288	JQ945184
B. arachidis CCBAU 33067	*Arachis hypogaea*	GU433528	GU 433503	GU433479	JX437673	JX437680	JX437666
B. arachidis CCBAU 23155	*Arachis hypogaea*	GU433524	GU 433500	G U433475	JX437671	JX437678	JX437664
B. genosp. alpha CIAT3101	*Centrosema plumieri*	AY591567	AY653774				
Bradyrhizobium sp. BuNoG5	*Glycine max*	EU574272	EU574192				
B. liaoningense ViHaR5	*Glycine max*	EU574327	EU574247				
B. genosp. TUXTLAS-5 1246v	*Vigna unguiculata*	FJ970345	FJ970398				
B. genosp. TUXTLAS-2 116m	*M. atropurpureum*	FJ970341	FJ970394				
B. genosp. TUXTLAS-15 1028v	*Vigna unguiculata*	FJ970329	FJ970382				
B. genosp. TUXTLAS-23 1722m	*M. atropurpureum*	FJ970355	FJ970408				
B. yuanmingense SR33	*Vigna radiata*	FJ514051	FJ514057				
B. genosp. TUXTLAS-11 8v	*Vigna unguiculata*	FJ970369	FJ970422				
B. genosp. TUXTLAS-9 1031v	*Vigna unguiculata*	FJ970330	FJ970383				
B. genosp. TUXTLAS-4 85v	*Vigna unguiculata*	FJ970368	FJ970421				
B. dagingense CCBAU 15768	*Glycine max*	HQ664969	HQ664972				
B. genosp. alpha BC-C1	*Glycine max*	AY591540	AY386761				
B. genosp. beta BRE-1	*Glycine max*	AY591551	AY599112				
B. genosp. SA3 BM25	*Vigna unguiculata*	EU364675	EU364651				
B. genosp. SA4 CB756	*Macrotyloma africanum*	EU364676	EU364653				
B. genosp. SA2 RC3b	*Vigna unguiculata*	EU364694	EU364671				
B. genosp. SA1 Ghiv	*Vigna unguiculata*	EU364682	EU364659				
B. genomic group G ICMP14754	*Acacia longifolia*	AY494832	AY494801				
B. elkanii BuNoR4	*Glycine max*	EU574276	EU574196				
B. canariense BC-MAM9	*Glycine max*	AY653747	AY653772				
B. genosp. TUXTLAS-1 1212v	*Vigna unguiculata*	FJ970342	FJ970395				
B. genosp. TUXTLAS-3 97m	*M. atropurpureum*	JN207432	JN207410				
B. genosp. TUXTLAS-7 1595v	*Vigna unguiculata*	FJ970351	FJ970404				
B. genosp. TUXTLAS-8 42v	*Vigna unguiculata*	FJ970363	FJ970416				
B. genosp. TUXTLAS-14 1064v	*Vigna unguiculata*	FJ970333	FJ970386				
B. genosp. TUXTLAS-16 114m	*M. atropurpureum*	FJ970340	FJ970393				
B. genosp. TUXTLAS-13 1809m	*M. atropurpureum*	FJ970359	FJ970412				
B. genosp. TUXTLAS-12 1648v	*Vigna unguiculata*	FJ970353	FJ970406				
B. genosp. TUXTLAS-10 141m	*M. atropurpurem*	FJ970350	FJ970403				
B. genosp. TUXTLAS-30 140m	*M. atropurpureum*	JN207433	JN207411				
B. genosp. TUXTLAS-22 1234V	*Vigna unguiculata*	FJ970344	FJ970397				
B. genosp. TUXTLAS-19 37m	*M. atropurpureum*	FJ970362	FJ970415				
B. genosp. TUXTLAS-20 66v	*Vigna unguiculata*	FJ970367	FJ970420				

(*continued*)

Table 3.2 (*Continued*)

Organism	Host Plant/ Isolation Source	*recA*	*glnII*	*atpD*	*gyrB*	*rpoB*	*dnaK*
B. genosp. TUXTLAS-21 107m	*M. atropurpureum*	FJ970334	FJ970387				
B. genosp. TUXTLAS-31 75m	*M. atropurpureum*	JN207434	JN207412				
B. genosp. TUXTLAS-18 1090v	*Vigna unguiculata*	FJ970335	FJ970388				
B. genosp. TUXTLAS-29 99m	*M. atropurpureum*	FJ970371	FJ970424				
B. genosp. ETH1 CIR42	*Crotalaria incana*	JQ809807	JQ809863				
B. genosp. ETH2 CIR1	*Crotalaria incana*	JQ809797	JQ809853				
Bradyrhizobium sp. IAR18B	*Indigofera arrecta*	JQ809823	JQ809879				
B. genosp. Aus1 WSM3976	*Acacia acuminata*	HE576604	HE576665				
B. genosp. Aus2 WSM3997	*Kennedia coccinea*	HE576625	HE576686				
B. genosp. Aus3 WSM3995	*Acacia acuminata*	HE576623	HE576684				
B. genosp. Aus4 WSM3977	*Gastrolobium capitatum*	HE576605	HE576666				
B. genosp. Aus5 WSM3981	*Gastrolobium capitatum*	HE576609	HE576670				
B. genosp. Aus6 WSM2238	*Bossiaea eriocarpa*	HE576600	HE576661				
B. genosp. Aus7 WSM2241	*Swainsona formosa*	HE576601	HE576662				
B. genosp. Aus8 ARR679	*Dunbaria singuliflora*	HE576586	HE576647				
B. genosp. Aus9 ARR549	*Flemingia parviflora*	HE576578	HE576639				
B. genosp. Aus10 ARR867	*Crotalaria* spesies	HE576599	HE576660				
B. genosp. Aus11 ARR858	*Tephrosia leptoclada*	HE576596	HE576657				
B. genosp. Aus12 WSM3985	*Acacia acuminata*	HE576613	HE576674				
B. genosp. Aus13 ARR751	*Crotalaria goreensis*	HE576593	HE576654				
B. genosp. Aus14 ARR595	*Desmodium* species	HE576581	HE576642				
B. genosp. Aus15 ARR862	*Uraria lagopodoides*	HE576598	HE576659				
B. genosp. Aus16 ARR696	*Galactia tenuiflora*	HE576589	HE576650				
B. genosp. Aus17 ARR410	*Acacia holosericea*	HE576573	HE576634				
B. genosp. Aus18 ARR401	*Desmodium* species	HE576572	HE576633				
B. genosp. Aus19 ARR375	*Vigna radiata*	HE576570	HE576631				
B. genosp. Aus20 ARR560	*Crotalaria montana*	HE576580	HE576641				
B. genosp. Aus21 ARR551	*Dunbaria singuliflora*	HE576579	HE576640				
B. genosp. Aus22 ARR487	*Acacia dimidiata*	HE576575	HE576636				
B. genosp. Aus23 ARR286	*Acacia mimula*	HE576567	HE576628				
B. genosp. Aus24 ARR312	*Crotalaria medicaginea*	HE576568	HE576629				
Bradyrhizobium sp. ORS278	*Aeschynomene sensitiva*	CU234118	CU234118	CU234118	CU234118	CU234118	CU234118
Bradyrhizobium sp. BTAil	*Beta vulgaris*	NC_009485	NC_009485	NC_009485	NC_009485	NC_009485	NC_009485
B. japonicum USDA 110	*Glycine max*	NC_004463	NC_004463	NC_004463	NC_004463	NC_004463	NC_004463
Bradyrhizobium sp. S23321	Paddy field soil*	NC_017082	NC_017082	NC_017082	NC_017082	NC_017082	NC_017082
Rhizobium etli CFN 42^T	*Phaseolus vulgaris*	CP000133	CP000133	CP000133	CP000133	CP000133	CP000133
R. leguminosarum USDA 2370^T	*Pisum sativum*	AJ294376	AF169586	AM418783	AM418830	AM295352	AM182081
Methylobacterium nodulans ORS2060^T	*Crotalaria* species	NC_011894	NC_011894	NC_011894	NC_011894	NC_011894	NC_011894
Rhodopseudomonas palustris BisB5	Marine, soils*	NC_007958	NC_007958	NC_007958	NC_007958	NC_007958	NC_007958
Rhodopseudomonas palustris CGA009	Marine, soils*	NC_005296	NC_005296	NC_005296	NC_005296	NC_005296	NC_005296
Rhodopseudomonas palustris HaA2	Marine, soils*	C_007778	NC_007778	NC_007778	NC_007778	NC_007778	NC_007778
Rhodopseudomonas palustris BisB18	Marine, soils*	C_007925	NC_007925	NC_007925	NC_007925	NC_007925	NC_007925
B. oligotrophicum LMG 10732^T	Rice paddy soils*	JQ619231	JQ619233				

*Source of non-nodule forming bacterial species.

B, *Bradyrhizobium*; R, *Rhizobium*; M, *Macroptilium*; genosp., genospecies; ^{T}, type strain of the species.

[http://www.ncbi.nlm.nih.gov/genbank] (Table 3.1). The sequences were aligned applying the MUSCLE software at EMBL–EBI (Edgar, 2004; Goujon et al., 2010), and the generated alignment was edited manually. The Tamura 3-parameter plus gamma distribution plus invariable site (T92 + G + I) was selected as the best-fit model of nucleotide substitution according to the Akaike Information Criterion, corrected (AICc) using the MEGA5 program (Tamura et al., 2011). The neighbor-joining (NJ) tree with 10,000 bootstrap replicates was constructed by the MEGA5 program.

3.2.2 Phylogenetic Analyses of *Bradyrhizobium* and the Related Taxa

Publicly available sequences of *recA*, *glnII*, *rpoB*, *atpD*, *dnaK*, and *gyrB* genes of all type strains of the currently described species of *Bradyrhizobium* except for *B. rifense* strain CTAW71 (which has only *recA* and *glnII* gene sequences), a type strain of *Methylobacterium nodulans*, and from the whole genome-sequenced *Bradyrhizobium* species and *Rhodopseudomonas palustris* were retrieved from the GenBank/EMBL database (http://www.ncbi.nlm.nih.gov/genbank/ and http://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html). In addition, widely common sequences, *recA* and *glnII* genes, were also collected for most root nodule forming unnamed genospecies of *Bradyrhizobium.* A list of strains and accession numbers of the sequences included in this study is shown in Table 3.2.

Sequences of each gene were aligned using ClustalW as implemented in Mega5 (Tamura et al., 2011). Phylogenetic trees were constructed from concatenated datasets of *dnaK _rpoB_gyrB_atpD_glnII5_recA* gene and *glnII_recA* gene sequences by NJ method using MEGA version 5 and with maximum-likelihood (ML) algorithms using PhyML 3.0 online programs (Dereeper et al., 2008). The NJ analyses were performed using the Kimura's 2-parameter distance correction (Kimura, 1980) model. The general time reversible with a gamma rate distribution and/or invariant site (GTR + G + I) nucleotide substitution model was used for ML tree constrictions because it was selected as the best-fit evolutionary model by jModelTest 0.1.1 program (Posada, 2008). The robustness of the ML topologies of *dnaK _rpoB_gyrB_atpD_glnII_recA* dataset was calculated with 100 bootstrap replications. Because *glnII_recA* dataset has a large number of taxa and takes a long time to compute with the bootstrap procedure, we used the faster approximate likelihood-ratio test (aLRT), which gives also branch support values very close to bootstrap values (Anisimova and Gascuel, 2006).

3.3 RESULTS

3.3.1 The *Agrobacterium–Rhizobium* Group

We constructed a 16S rRNA phylogenetic gene tree based on sequences deposited in GenBank representing mainly 160 taxonomic type strains. The strains were chosen based on the taxa listed on the taxonomy website [http://edzna.ccg.unam.mx/rhizobial-taxonomy/node/2]. On this website, pathogenic *Agrobacterium* species and nodulating alpha- and betaproteobacteria (rhizobia) as well as closely related taxa are listed. The reader is referred to this website for references for species descriptions. From this page, there are links to the website list of prokaryotic names with standing in nomenclature, which is maintained by Euzéby (2013). From this site, there are again links to the published taxonomic descriptions. The expansion of the taxonomy in this part of the phylogenetic tree has recently been very fast, and new species are described almost every month.

The genus name *Agrobacterium* traditionally meant plant pathogenic bacteria harboring Ti or Ri pathogenicity plasmids, but the genus also included nonpathogenic strains related to pathogenic strains. In a similar manner, genus *Rhizobium* stood for N-fixing species harboring symbiotic plasmids or islands. Since accessory elements (plasmids, symbiotic islands) encode niche adaptation and confer unstable properties to their host bacteria depending on the presence or absence of the elements, those should not have preference when naming species and genera. The transition from a pronounced, plant phenotype-based taxonomy to one based on the evolution of the more stable core genome has been slow.

There are two main reasons for changing genus designations. One is based on observations of evolution leading to speciation in the core genome. The other is the practical need to split genera in order to better be able to use the names when communicating and thus create new names for monophyletic clades deserving to be named (Lindström and Gyllenberg, 2006). The first well-founded new genus that arose from the *Agrobacterium–Rhizobium* group was *Bradyrhizobium* in 1982 [http://www.bacterio.cict.fr/b/bradyrhizobium.html]. The phylogeny and taxonomy of genus *Bradyrhizobium* is further elaborated on in Figures 3.4 and 3.5 and will be discussed later.

3.3.2 The *Mesorhizobium* Clade

Mesorhizobium was separated from the rest of the rhizobial genera in 1997 [http://www.bacterio.cict.fr/m/mesorhizobium.html]. It forms a well-supported clade located distantly from other rhizobial genera. However,

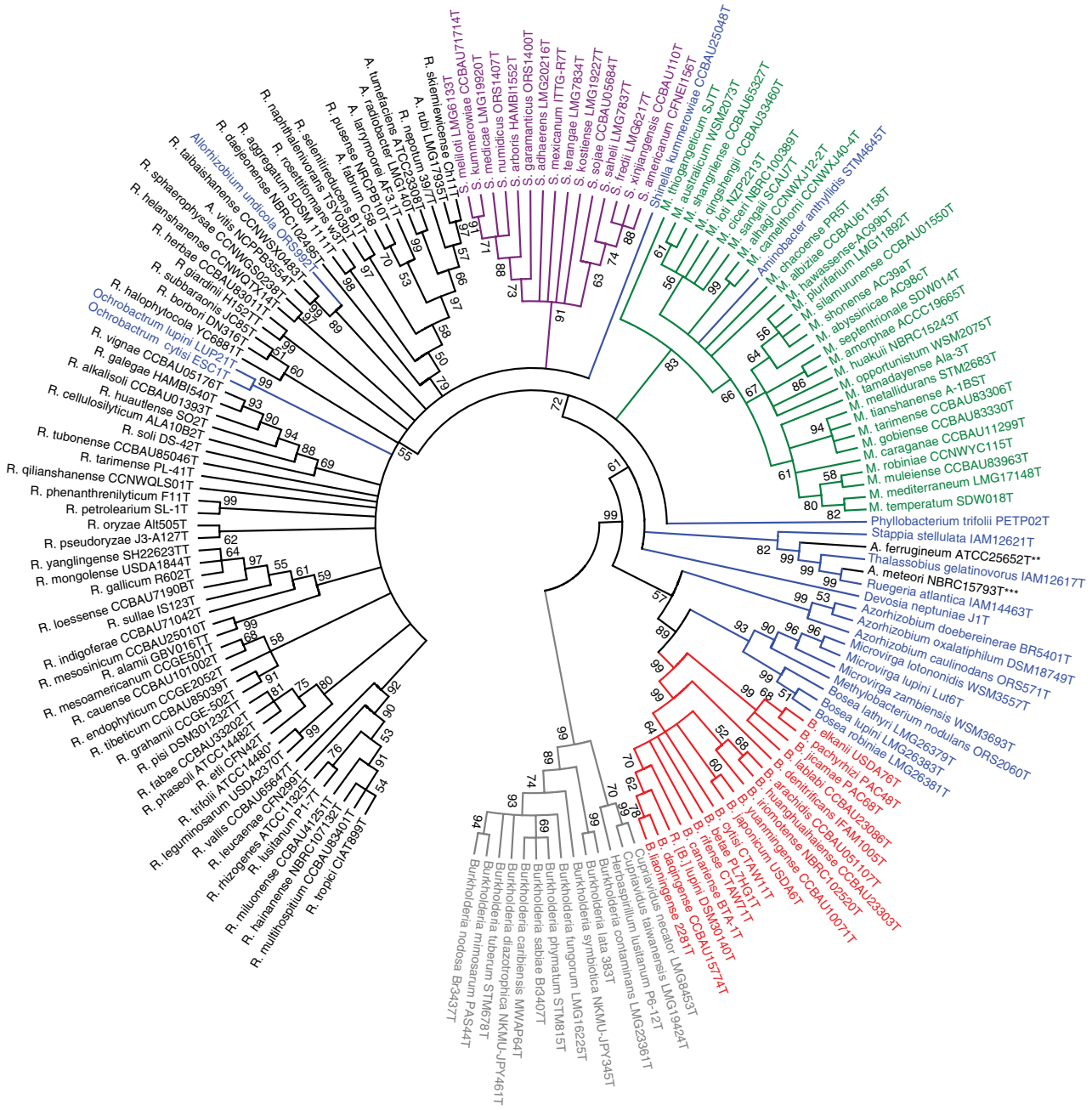

Figure 3.3 The phylogenetic tree based on 16S rRNA gene sequences, illustrating the relationships among 160 proteobacterial species. The neighbor-joining tree was constructed with 10,000 bootstrap replicates, and the cut-off is 50%. The type strains are shown by a "T" at the end of each strain code. The genus names are abbreviated as follows: *A.*, *Agrobacterium*; *B.*, *Bradyrhizobium*; *M.*, *Mesorhizobium;* *R.*, *Rhizobium*; and *S.*, *Sinorhizobium*. Other genera of *Alphaproteobacteria* are in blue, and the *Betaproteobacteria* are in gray. Alternative names: **Rhizobium leguminosarum*, ***Pseudorhodobacter ferrugineus*, ****Agrobacterium atlanticum*.

in 2012, "*Aminobacter anthyllides*" was described as a nodulating species. So far this species was not validly described [http://www.bacterio.cict.fr/nonvalid.html], but once it is validated it is possible to propose that *Mesorhizobium* and *Aminobacter* are merged and then the name *Aminobacter*, published in 1992, will be the name to use according to the Bacteriological Code [http://www.bacterio.cict.fr/a/aminobacter.html]. A valid description is one that was published in the *IJSEM*. Species descriptions published in other journals must be published in a validation list in

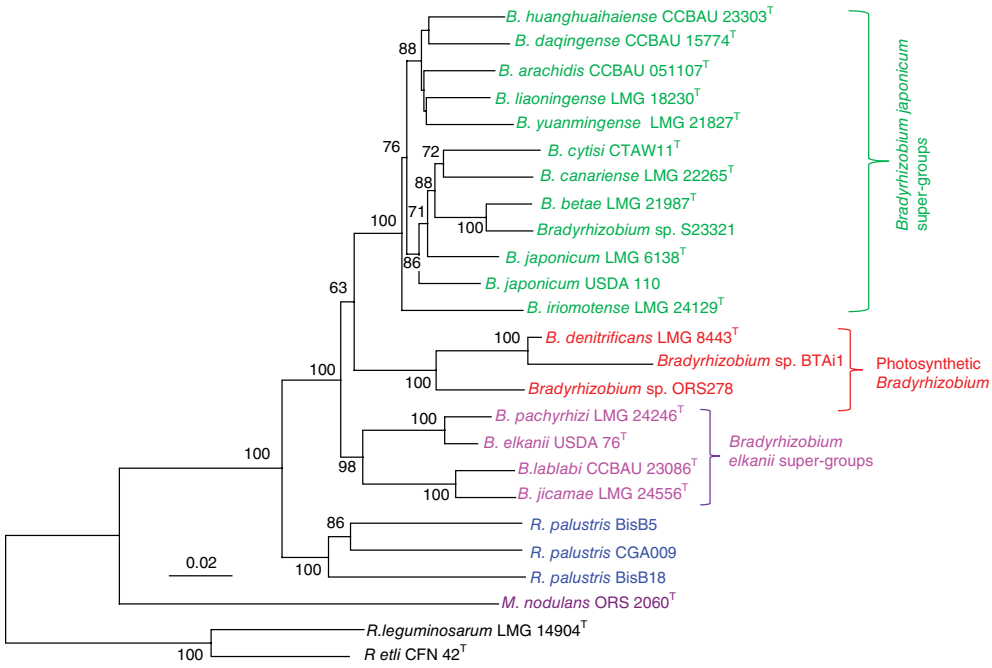

Figure 3.4 Maximum-likelihood (ML) phylogenetic tree constructed based on concatenated *dnaK_rpoB_gyrB_atpD_glnII_recA* gene sequences (total length 2773 bp including gaps), showing the relationships among root-nodulating bacterial species in the genus *Bradyrhizobium* (see also Chapter 18). The tree includes all described type strains of nitrogen-fixing bacterial species of the genus *Bradyrhizobium* except *B. rifense* strain CTAW71. In addition, the whole genome-sequenced *Bradyrhizobium* species, the type strain of *Methylobacterium nodulans*, and *Rhodopseudomonas palustris* were also included in the tree. The tree is rooted with the type strains of *Rhizobium leguminosarum* and *R. etli*, and the type strains are indicated with ^{T}. ML bootstrap support >50% indicated at each node.

IJSEM. Until validation, the name is put within quotation marks.

3.3.3 The *Sinorhizobium* Clade

The genus name *Sinorhizobium* was proposed in 1988 for a new species, *S. xingjiangense*, isolated in China [http://www.bacterio.cict.fr/s/sinorhizobium.html]. The taxonomic community was not convinced by the work. In 1994, however, the monophyletic clade carrying among other novel species of the previously described *S. xingjiangense* was proposed to form a new genus, which according to the rules of taxonomy should be *Sinorhizobium.* As the model species for molecular studies, *Rhizobium meliloti*, was a member of the newly formed clade, *Rhizobium* biologists surprisingly happily started using the new name *Sinorhizobium meliloti. Sinorhizobium* is a monophyletic clade branching out from the large *Agrobacterium–Rhizobium* group. It now carries 15 nodulating species, most of which are well studied. Using the name *Sinorhizobium* is phylogenetically sound. A problem arose when it was revealed that a species called *Ensifer adhaerens* was in fact phylogenetically *Sinorhizobium.* Why was this problematic? Since the nonsymbiotic *Ensifer* (1982) [http://www.bacterio.cict.fr/e/ensifer.html] had been published as a name before *Sinorhizobium*, all species of the genus *Sinorhizobium* had to be transferred to the genus *Ensifer*, in line with rule 38 of the Bacteriological Code. This fact causes a lot of confusion in the literature and in databases. In this chapter, we use the synonym *Sinorhizobium* for *Ensifer.*

3.3.4 Other Clades and Species in the *Agrobacterium–Rhizobium* Group

There are more clades and species to be sorted out in the *Agrobacterium–Rhizobium* group. *Agrobacterium* [http://www.bacterio.cict.fr/a/agrobacterium.html] is intermingled with *Rhizobium* [http://www.bacterio.cict.fr/r/rhizobium.html]. This led Young et al. (2001) to propose that all species in this group should be called *Rhizobium*. Contrary to Figure 3.3 and many other phylogenies, *Sinorhizobium* was placed outside the *Rhizobium clade* in their phylogenetic reconstruction. As a consequence, most authors now use the name *Rhizobium rhizogenes* instead of the former *A. rhizogenes*, which is located close to *R. tropici* in the 16S rRNA gene tree. The branches from *R. aggregatum* to *R. skierniewicense* carry species with genus name *Agrobacterium* or *Rhizobium*, reflecting the confusion caused by Young et al. (2001) by calling the whole group *Rhizobium.*

Figure 3.5 Maximum-likelihood (ML) phylogenetic tree constructed with concatenated *glnII* (516 bp)_*recA* (417 bp) gene sequences, showing the relationships among mainly root-nodulating bacterial species and genospecies of the genus *Bradyrhizobium* (see also Chapter 18). In addition, whole genome-sequenced *Bradyrhizobium* species, a type strain of *Methylobacterium nodulans,* and *Rhodopseudomonas palustris* were also included in the tree. The tree is rooted with the type strains of *Rhizobium leguminosarum* and *R. etli*, and the type strains (green) are indicated with ^{T}. ML approximate likelihood-ratio test supports >70% indicated at each node.

The plant pathogenic *R. skierniewicense*, *A. rubi*, *A. nepotum*, *A. radiobacter*, *A. larrymoorei*, and "*A. fabrum*" strain C58 (Lassalle et al., 2011) are positioned within a larger clade in Figure 3.3 with nonpathogenic, nonsymbiotic *R. pusense*, *R. selenitireducens*, *R. naphtalenivorans*, *R. rosettiformans*, and *R. aggregatum*. We think that this clade is large and defined enough to warrant it a genus name different from *Rhizobium*, though further phylogenetic studies with more housekeeping genes are needed to verify this assumption. The symbiotic *R. daejeonense* is in the same clade though the most distant member of it.

Allorhizobium undicola and *Agrobacterium vitis* with the very close *Rhizobium taibaishanense* form another well-separated clade. *A. vitis* is pathogenic, *A. undicola* is symbiotic, and *R. taibaishanense* has no plant-related phenotype reported.

R. sphaerophysae with "*R. helanshanense*" (not validly published), *R. herbae* with *R. giardinii*, as well as *R. subbaronis* with *R. borbori* and *R. halophytocola* form small, well-separated clades in the tree (Fig. 3.3). Of those, *R. sphaerophysae*, *R. herbae*, *R. giardinii*, and *R. helanshanense* were reported as symbiotic.

Ochrobactrum cytisi isolated from *Cytisus scoparius* and *O. lupini* from *Lupinus albus* are symbiotic, but probably only minor symbionts.

Rhizobium vignae, *R. galegae*, *R. alkalisoli*, and *R. huautlense* form a clade of symbiotic bacteria with neighbors *R. cellulosilyticum* (minor symbiont) and nonsymbiotic *R. soli.*

R. tubonense and "*R. qilianshanense*" (unvalidated) were isolated from nodules and form well-separated branches in the 16S rRNA gene tree. *R. tarimense* is a recent addition to this group and presumably nonsymbiotic. The symbiotic *R. petrolearum* and nonsymbiotic "*R. phenanthrenilyticum*" (unvalidated) form another well-separated clade, as do the symbiotic *R. oryzae* and the nonsymbiotic *R. pseudoryzae.*

The symbiotic *R. gallicum*, *R. mongolense*, and *R. yanglingense* strains form a monophyletic lineage with very low genetic diversity, as pointed out by Vinuesa et al. (2005), who based on multilocus analysis suggested that they form just one species. This change of names has not formally been done, which is a pity. However, their study emphasized the need for proper phylogenetic reconstructions with several housekeeping genes to detect speciation and delineate species.

R. loessense, *R. sullae*, *R. indigoferae*, *R. mesosinicum*, and *R. alamii* are symbiotic species in the same clade. The *R. indigoferae* type strain has been distributed as two different genotypes, the second clustering with *R. leguminosarum*.

The next larger clade consists of the symbiotic *R. mesoamericanum*, *R. tibeticum*, and *R. grahamii*. "*R. cauense*" and *R. endophyticum* were isolated from plant tissue (*Kummerowia stipulacea* nodule; *Phaseolus* bean tissue, respectively). The type strain of *R. endophyticum*, isolated as a bean endophyte, became fully symbiotic when the symbiotic plasmid of *R. tropici* was introduced to it (López-López et al. 2010).

The *R. leguminosarum* "superclade," which was recognized by Aserse et al. (2012a), is also visible in the 16S tree. It consists of symbiotic, well-characterized bacteria. This clade encompasses the *R. leguminosarum* type strain, the very closely related *R. fabae* and *R. pisi*, as well as *R. phaseoli*, *R. etli*, and *R. leguminosarum* symbiovar trifolii, the former type strain of *R. trifolii*, which is not a recommended species name any longer.

Another "superclade" consists of the symbiotic *R. tropici*, *R. leucaneae*, *R. lusitanium*, *R. miluonense*, *R. hainanense*, *R. multihospitum*, and *R. vallis*, as well as the pathogenic *R. rhizogenes*.

The phylogenetic branches from *Phyllobacterium trifolii* to *Bosea lupine* represent a mix of genera and species with diverse properties. *P. trifolii* was reported as symbiotic [http://www.bacterio.cict.fr/p/phyllobacterium.html] on clover and lupine. *Devosia neptuniae* [http://www.bacterio.cict.fr/d/devosia.html] and the *Azorhizobium* [http://www.bacterio.cict.fr/a/azorhizobium.html] and *Microvirga* [http://www.bacterio.cict.fr/m/microvirga.html] species as well as *M. nodulans* [http://www.bacterio.cict.fr/m/methylobacterium.html] were reported as symbiotic and demonstrate the taxonomic diversity of symbiotic features within the alphaproteobacteria. These bacteria represent some interesting symbiotic and physiological features, as *Azorhizobium* is both stem nodulating and known to fix N *ex planta* and *Methylobacterium* consumes several one-carbon substrates. The *Bosea* [http://www.bacterio.cict.fr/b/bosea.html] species were described as endophytic but nonsymbiotic. The other species in this group were previously (and still are) erroneously described as *Agrobacterium* species and they are all aquatic bacteria.

3.3.5 The Betaproteobacteria

The betaproteobacteria in Figure 3.3 are all reported as symbiotic (see also Chapter 89). The *Burkholderia* species represent a fraction of all species in this large genus [http://www.bacterio.cict.fr/b/burkholderia.html], whereas *Cupriavidus taiwanensis* is the only symbiotic species in the genus, which otherwise displays a collection of species with diverse properties [http://www.bacterio.cict.fr/c/cupriavidus.html]. *Herbaspirillum* [http://www.bacterio.cict.fr/h/herbaspirillum.html] species are often plant associated and *H. lusitanium*, isolated from a *Phaseolus* bean nodule, is endophytic but not symbiotic.

Gyaneshwar et al. (2011) have summarized features of the symbiotic betaproteobacteria in a recent comprehensive review (see also Chapter 17). These bacteria were

so far found to infect mimosoid legumes in South America and Asia (*Mimosa* sp.), papilionoid legumes in South Africa (*Aspalathus*, *Cyclopia*), and some native papilionoid legumes in Australia. Some strains displayed promiscuity infecting, for example, *Phaseolus* bean. For scientists working with *Burkholderia*, it is important to distinguish symbiotic from pathogenic species, because a genus name that refers to pathogenic bacteria will cause problems in society.

3.3.6 The Genus *Bradyrhizobium*

Genus *Bradyrhizobium* for a long time had only two described species: *B. japonicum* and *B. elkanii*. The current list of taxa is much longer, and new species are described at an increasing pace. Some patterns are thus starting to emerge (Fig. 3.4; see also Chapter 18). As the 16S rRNA gene is not variable enough among bradyrhizobia to be useful for species delineations, other housekeeping genes should be used to produce reliable phylogenies. In Figure 3.4, a phylogenetic tree based on concatenated *dnaK_rpoB_gyrB_atpD_glnII_recA* gene sequences is shown. Three supergroups are highlighted, representing *B. japonicum* – and *B. elkanii* –related species and the group containing photosynthetic strains. Most species in the former groups are nodulating (exception *B. betae*), *Bradyrhizobium* sp. strains BTAi1 and ORS278 are photosynthetic and stem nodulating on *Aeschynomene* species, but lack the canonical *nod* genes (Giraud et al. 2007). *Bradyrhizobium denitrificans*, the former *Blastobacter denitrificans*, is also able to form root and stem nodules on some *Aeschynomene* species. The nonsymbiotic *Blastobacter oligotrophicum* is also a member of this clade (Fig. 3.5).

Figure 3.5 shows a more complex pattern. In addition to the supergroups delineated in Figure 3.4, two unnamed *Bradyrhizobium* deep lineages are pointed out. They represent isolates from the Australian continent (Stepkowski et al., 2012). Furthermore, many unnamed species, denoted as genospecies, are occupying branches in the tree. These isolates came from Australia (Aus) (Stepkowski et al., 2012), Ethiopia (ETH) (Aserse et al., 2012a), Mexico (TUXTLAS) (Ormeño-Orrillo et al., 2012), South Africa (SA) (Steenkamp et al., 2008), and the Asian continent (Vinuesa et al., 2008). The authors who discovered these have chosen not to name the species, but they represent interesting novel biodiversity.

Other authors have rushed to describe a new species. The novel "*B. arachidis*" species displays within species diversity. In Figure 3.5, it seems to represent two lineages, each one with members also from other genospecies. In the paper by Wang et al. (2013), the 16S and concatenated housekeeping gene sequences are only compared with those representing described species. This is unfortunate, since a Blast search could have retrieved also sequences representing published genospecies.

3.4 DISCUSSION

The results presented clearly demonstrate that there is a discrepancy between population genetic and phylogenetic studies, on the one hand, and taxonomy on the other. Population genetics requires several strains to be studied, and speciation can be detected in phylogenetic reconstructions only when enough strains and genes are used, whereas current taxonomic practice encourages scientists to put a minimum of effort into the selection of a representative set of strains and genes. Instead, even one single strain is enough if all tests requested by the editors of the *IJSEM* are performed to describe a new species.

Graham et al. (1991) published the paper "Proposed minimal standards for the description of new genera and species of root- and stem-nodulating bacteria," which expressed the views of the ICSP subcommittee at that time. Those guidelines are partly still valid, but many authors follow them too strictly. Phylogenies of housekeeping genes are often presented nowadays, and this is a step in the right direction. In the minimal standards, the host plants for the species that were described to that date (1991) were proposed to be used in plant tests for putative new species. Those plants are *Medicago sativa, Pisum sativum, Phaseolus vulgaris, Trifolium repens, Lotus corniculatus, Glycine max, Vigna unguiculata, Leucaena leucocephala, Macroptilium atropurpureum,* and *Galega officinalis.* Host plants known to be nodulated by the strains being tested should be included as well as the host plant from which each strain was isolated. Today, those plants are partly excessive and the selection of plants should be given proper thought.

Nowadays, the phylogeny of *nif* and *nod* genes is also often reported for new species. Since the species delineations are based on core/housekeeping gene phylogenies, but symbiotic properties of rhizobia are important for biological and practical reasons, symbiovars can be designated that refer to plant nodulation and N fixation phenotype as well as genotype. Rogel et al. (2011) listed all currently described symbiovars. Species that symbiovars have been designated for are generally well described also in terms of biological properties.

We have used the symbiotic or pathogenic to describe the phenotypes associated with the described species. If we consider several of the branches in Figure 3.3, symbiotic or pathological properties are often incompletely tested and described. Lindström et al. (2010) reviewed properties of symbiotic bacteria in the field and processes shaping their genomes. It must be borne in mind that phenotypic properties associated with many of the described species are only snapshots of dynamic genomes. The example of the

type strain of *R. endophyticum*, isolated as a bean endophyte, which became fully symbiotic when the symbiotic plasmid of *R. tropici* was introduced to it (López-López et al., 2010), demonstrated the plasticity of the genomes and the role of accessory genes in shaping them. It would be desirable that taxonomists took some more interest in these aspects, adding an evolutionary dimension to the species descriptions.

As pointed out in the introduction, symbiotic genes are inherited horizontally and vertically. Current knowledge is mainly based on properties of the alphaproteobacterial rhizobia. It is thus still a matter of speculation whether the *nif* genes of alphaproteobacteria were inherited from the betaproteobacteria, and the *nod* genes were transferred in the other direction or vice versa (Masson-Boivin et al., 2010; Gyaneshwar et al., 2011). More studies in this direction will add to the excitement of taxonomy as well.

There is certainly room for revision of genera and species described in this paper. Especially the *Agrobacterium–Rhizobium* group calls for thorough phylogenetic and population genetic studies of the clades outlined in Figure 3.3. We anticipate that *Agrobacterium* will be maintained for the clade with *Agrobacterium tumefaciens. A. vitis* on the contrary might be merged with *A. undicola* and *R. taibaishanense*. This clade displays a diversity of plant phenotypes and should be properly explored. *Rhizobium galegae* and the related taxa are in focus in our laboratory and we hope to present a taxonomic revision soon.

Nowadays full genome sequences for bacteria are easy to obtain. We propose that all type strains should have their genome sequence determined before publication. This would enable interesting comparisons between species and across taxa and would advance our understanding of the biology of these interesting plant-interacting prokaryotes.

NOTE

Two new genera, *Neorhizobium and Pararhizobium*, have been described for the family *Rhizobiaceae* since 2014 (Mousavi et al., 2014, 2015). The 16 new species combinations that were described in the family *Rhizobiaceae* in 2014 and 2015 are listed as follows: *Neorhizobium galegae, Neorhizobium huautlense, Neorhizobium alkalisoli, Agrobacterium nepotum, Agrobacterium pusense, Agrobacterium skierniewicense, Allorhizobium vitis, Allorhizobium taibaishanense, Allorhizobium paknamense, Allorhizobium oryzae, Allorhizobium pseudoryzae, Allorhizobium borbori, Pararhizobium giardinii, Pararhizobium capsulatum, Pararhizobium herbae, and Pararhizobium sphaerophysae.*

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Chapter 4

Evolution of *Rhizobium* Nodulation: From Nodule-Specific Genes (Nodulins) to Recruitment of Common Processes

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4.1 NODULE FORMATION IN A NUTSHELL

The formation of the root nodule is initiated at the epidermis of plant roots where signals of the bacteria are perceived. In response to the signal, among others, root hairs redirect their growth toward the bacterium. In this way, a curl is formed around the dividing bacteria that become entrapped in a pocket inside the curl. After completion of the curl, an infection thread is initiated starting from this pocket. This infection thread is a tubular invagination of the plasma membrane of the plant, filled with rhizobia, that grows inside the root hair toward the root cortex. While the infection thread grows inwards, cells of the cortex start to divide to form the nodule primordium from which the nodule develops (Ferguson et al., 2010; Hirsch, 1992; Luyten and Vanderleyden, 2000; see also Chapters 41, 50, 51).

4.2 SIGNALING IN SYMBIOSIS

To establish a symbiotic interaction, the two partners need to exchange signals as a biochemical handshake. For the *Rhizobium*–legume symbiosis, the signaling is mainly studied during early steps in the epidermis. The signal that sets the nodulation process in motion is the Nod factor. This is a decorated lipochito-oligosaccharide (LCO) that is perceived by two Nod factor receptors. These are the LysM receptor kinases LYK3/NFR1 and NFP/NFR5 in *Medicago truncatula* and *Lotus japonicus,* respectively (Arrighi et al., 2006; Limpens et al., 2003; Radutoiu et al., 2003; Smit et al., 2007; see also Chapter 51). The receptors activate the so-called common signaling pathway. Not only is this pathway, as its name suggests, unique to the *Rhizobium*–legume symbiosis, but it is also used in the mycorrhizal symbiosis (Catoira et al., 2000; Geurts and Bisseling, 2002; Hocher

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et al., 2011). The common signaling pathway starts with the LRR-type receptor DMI2 (Limpens et al., 2005) and the putative cation channel DMI1 (Ane et al., 2004). Downstream of DMI1 and DMI2 act two nucleoporins: NUP133 and NUP85 (Kanamori et al., 2006; Saito et al., 2007). All these components are essential for the induction of nuclear calcium oscillations (Peiter et al., 2007; Wais et al., 2000; see also Chapters 54, 57). These oscillations are interpreted by the calcium and calmodulin-dependent kinase CCamK/DMI3 (Levy et al., 2004; Mitra et al., 2004; Oldroyd and Downie, 2004). CCamK and its interactor Cyclops/IPD3 are the last components of the common signaling pathway (Messinese et al., 2007; Ovchinnikova et al., 2011; Yano et al., 2008) and activate transcription factors that regulate a wide range of genes, which initiate the formation of a nodule and ultimately the formation of the symbiotic interface (see later).

4.3 THE INDETERMINATE NODULE

In legumes, two types of nodules can be distinguished: determinate and indeterminate nodules. Determinate nodules are formed on, for instance, the model legume *L. japonicus* and soybean. In these nodules, the meristem is transiently present and as a result the formation of infected cells, and so the symbiotic interfaces, are formed more or less simultaneously (Ferguson et al., 2010). Therefore, the subsequent developmental stages of symbiotic interface formation are rather difficult to study in determinate nodules.

Indeterminate nodules are formed among others on the model organism *M. truncatula*, pea, alfalfa, clover, and vetch. These indeterminate nodules contain a nodule meristem at their apex that continuously adds new cells to the nodule. Therefore, in these nodules, a series of subsequent developmental stages are present along their longitudinal axis. This facilitates research on the development of the symbiotic interface. The indeterminate nodule has four developmental zones: the meristem, the infection zone, the fixation zone, and the senescent zone. The meristem continuously provides the nodule with new cells. In the infection zone, the cells derived from the meristem are continuously infected by rhizobia, which are released from an infection thread that penetrates these cells. In indeterminate nodules, in general, individual bacteria are surrounded by a host-derived membrane. The bacterium with the plant-derived membrane is called symbiosome (Fig. 4.1a; see also Chapter 68). The symbiosomes continue to divide and develop into a mature state where they fix atmospheric nitrogen into ammonia in return for carbohydrates. This zone, where the rhizobia fix nitrogen and the symbiotic interface is completed, is called the fixation zone. Finally, in the senescent zone, symbiosis is terminated by the plant by fusion of symbiosomes with lytic vacuoles (D'Haeze and Holsters, 2002; Downie, 2007; Esseling et al., 2003; Ferguson et al., 2010; Hirsch, 1992; Jones et al., 2007; Murray, 2011).

4.4 SIGNALING INSIDE THE NODULE

Inside the nodules, recognition of the rhizobial Nod factor might be essential for the formation of the symbiotic interface, although this has not yet been demonstrated. In the nodules, the Nod factor receptors and other components of the signaling cascade are expressed at the apex (Limpens et al., 2005). However, attempts to visualize the Nod factor receptors in nodules failed (Haney et al., 2011; Madsen et al., 2011). Furthermore, a functional analysis of the Nod factor receptors in nodules is not available. However, some components of the Nod factor signaling cascade have been shown to control the bacterial release from infection threads and so the formation of the symbiotic interface (see also Chapter 59). Knockdown of DMI2 or IPD3 (the interactor of DMI3) blocks the formation of the symbiotic interface (Limpens et al., 2005; Ovchinnikova et al., 2011). When the *Medicago* DMI3 mutant is complemented with the DMI3 homolog from rice a nodule is formed, but release of the bacteria is blocked (Chen et al., 2007). This suggests that DMI3 is also an essential component for the release of rhizobia. How the signaling cascade is activated remains to be demonstrated, but Nod factors might play a role. The rhizobial genes essential for the production of Nod factors are active in the infection zone of root nodules (Schlaman et al., 1991; Sharma and Signer, 1990). Furthermore, rhizobia unable to produce Nod factors inside the nodules are not released (Marie et al., 1994). Whether and how Nod factors are perceived in root nodules and whether this results in the activation of the Nod factor signaling cascade remains to be demonstrated. Rhizobia are checked for their Nod factor profile/signature by the entry receptor in curled root hairs (Ardourel et al., 1994; Catoira et al., 2001; Smit et al., 2007). Nevertheless, a final Nod factor structure checkpoint would contribute to the maintenance of the symbiotic nature of the interaction.

4.5 *RHIZOBIUM* SYMBIOSIS IN *PARASPONIA*

Insight into the evolution of the legume–*Rhizobium* symbiosis can be obtained by a comparison with other (non-) legume endosymbioses. First, we make a comparison with the *Rhizobium–Parasponia* symbiosis. The symbiosis with rhizobia is almost completely restricted to legumes. The only exceptions are tropical trees belonging to the genus *Parasponia* that also can form an interaction with rhizobia. *Parasponia* is a part of the Celtidacea and so

Figure 4.1 Symbiotic interfaces are formed in several symbiotic interactions. (a) In legumes, the symbiosis with rhizobia leads to the formation of intracellular symbiosomes. (b) Formation of fixation threads in *Parasponia* in symbiosis with rhizobia. (c) Formation of arbuscules in symbiosis with arbuscular mycorrhizal fungi. (d) In pathogenic biotrophic fungi, interaction leads to the formation of haustoria.

only remotely related to legumes. Therefore, this symbiosis evolved independently. *Parasponia*, such as the legumes, forms a nodule to house the bacteria. These *Parasponia* nodules are modified lateral roots. This nodule has a central vascular bundle, no root cap, and the bacteria are hosted in the expanded cortex.

The infection thread penetrates nodule cells, but rhizobia are not released from the infection thread. Instead, fixation threads form a continuum with the infection thread. Similar to infection threads, the fixation threads are bound by a cell wall albeit it is markedly thinner than the wall of an infection thread (Fig. 4.1b; see also Chapter 57). So a clear difference between fixation threads and symbiosomes is the presence of this thin cell wall, which is absent in symbiosomes (Webster et al., 1995). The rhizobia fix nitrogen in the fixation threads and must get carbohydrates in return. This symbiotic interface of *Parasponia* is similar to the symbiotic interface formed in primitive legumes such as *Andira* spp. and many species belonging to the Fabaceae with subfamily Caesalpinioideae (Defaria et al., 1989).

Research on *Parasponia* has been recently revitalized and can now make use of the achievements obtained with model legume systems. It has not yet been studied whether the common signaling pathway is involved in *Parasponia*. However, studies on one of the Nod factor receptors provided insight into the evolution of these receptors. To prove that also in *Parasponia* the Nod factor is the signal that starts symbiosis, Op den Camp et al. (2011) knocked down the *Parasponia* homolog of the Nod factor receptor NFP. This led to a marked (90%) reduction of the nodule number. Thus, although evolved independently, the same signal molecule is recruited to induce this nonlegume nodule symbiosis. This points to an important constraint in nodule evolution. This experiment revealed an important role of Nod factor perception. In the nodules that are formed, the knockdown of NFP specifically blocked the formation of fixation threads. So it proved that Nod factors need to be perceived to form a symbiotic interface in *Parasponia*, and this strongly suggests that this is also the case in legumes which is in line with the important role of the common signaling pathway in the formation of the interface.

Parasponia acquired nodulation rather recently. This makes *Parasponia* and the closely related non-nodulating *Trema* an ideal biological system to study the evolutionary origin of nodulation as the amount of evolutionary noise is lower than in legumes.

4.6 ACTINORHIZAL N-FIXING SYMBIOSIS

Another N-fixing nodule symbiosis is the interaction of actinorhizal plants (e.g., alder, *Casuarina*, bayberry, and sweet fern) and (Gram-positive) actinobacteria of the genus *Frankia*. The root nodule that is formed is a modified lateral root such as the *Parasponia* nodules. In these nodules, the bacteria are also hosted intracellularly. The hyphae of *Frankia* enter the plant by an infection thread-like structure. Inside the nodule, these hyphae branch extensively to fill the entire nodule. As in *Parasponia*, the bacteria are not released from the thread: a vegetative hyphae surrounded by a plant membrane is formed (Fig. 4.1b). The membrane surrounding the hyphae is the symbiotic interface. From the tip of these vegetative hyphae, symbiotic vesicles are formed in which nitrogen fixation takes place (Berg et al., 1999; Pawlowski and Demchenko, 2012; Wall, 2000; see Chapters 35, 42, 43, 48, 55).

A knockdown of the SymRK homolog, a component in the common signaling pathway, blocks the formation of vegetative hyphae in *Casuarina*. Although other components of the common signaling pathway have not been tested, this suggests that the common signaling pathway (also known as CSSP or SYM) is essential for the formation of the symbiotic interface in *Casuarina* (Gherbi et al., 2008; Markmann et al., 2008; see Chapter 55). The nature of the signal molecule initiating *Frankia* symbiosis is unknown. However, the fact that the common signaling pathway seems involved suggests a shared evolutionary origin with the *Rhizobium* symbiosis.

4.7 ARBUSCULAR MYCORRHIZAL SYMBIOSIS

In comparison with the *Rhizobium*–legume and the actinorhizal symbiosis, the AM symbiosis has a much broader host range. Approximately 80% of all land plants can interact with AM fungi. The rhizobial and actinorhizal symbioses are also much younger (~60–80 million years) compared to the AM symbiosis (475 million years). The symbiosis with AM fungi provides the plant with an improved uptake of, for example, water, phosphate, and nitrogen. It is thought that this symbiosis was important for the colonization of land by plants (Hata et al., 2010). The AM symbiosis starts off with the germination of a fungal spore. Upon contact with the plant epidermis a hyphopodium is formed to allow the fungus to penetrate the root. When the fungus reaches the root cortex, it spreads longitudinally. The fungus forms a trunk that penetrates the plant cell. This trunk is still surrounded by a cell wall. From the trunk arbuscules are formed; highly branched intracellular hyphae are surrounded by a host membrane and lack a cell wall. This periarbuscular membrane is the symbiotic interface (Fig. 4.1c) (Genre et al., 2005; Hata et al., 2010; Ivanov et al., 2010). So in this way, AM fungi, rhizobia, and *Frankia* are hosted in a similar intracellular manner.

Similar to rhizobia, the mycorrhizal fungi produce signal molecules that induce symbiosis (Myc factors). One of these Myc factors has a remarkably similar structure as the rhizobial Nod factor: both are LCOs (Maillet et al., 2011; see Chapter 51). Furthermore, chitin tetramers and pentamers are produced by AM fungi. These are shorter than the chitin fragments inducing defense responses and do not trigger defense responses either (Genre et al., 2013). For defense responses, chitin octamers are needed to facilitate receptor dimerization (Liu et al., 2012). The tetramers and pentamers are not able to facilitate receptor dimerization. Interestingly, the Nod factor and LCOs have a backbone of four chitin residues. The short-chain oligomers can, like LCOs, induce nuclear calcium spiking in *Medicago* via the common signaling pathway (see Chapter 54). The receptors that recognize these AM fungal LCOs and chitin oligomers in legumes are not known. In contrast to *Parasponia* (Op den Camp et al., 2011), legume Nod factor receptors are not needed to establish a symbiosis with AM fungi (Radutoiu et al., 2003).

The legume Nod factor receptors belong to gene families where, for example, NFP is often a single copy "family" in nonlegumes. Therefore, it seems probable that at least the fungal LCOs are perceived by receptors that are closely related to Nod factor receptors. In legumes, these have probably diverged by gene duplication and neofunctionalization (Zhang et al., 2007). So most likely, both the Nod factors, its receptor and the common signaling pathway, have an evolutionary origin in the mycorrhizal symbiosis.

In both the *Rhizobium* nodule and the AM fungal symbiosis, a cell-wall-free symbiotic interface is formed. In the *Rhizobium* symbiosis this is the membrane around the symbiosome, and in the AM symbiosis the periarbuscular membrane. Ivanov et al. (2012) identified in *Medicago* a specific exocytotic pathway that is required for the formation of the symbiotic interface by studying the role of vesicle-associated membrane proteins (VAMPs). VAMPs are essential for exocytosis. VAMPs belong to the SNARE proteins. These proteins guide the fusion of vesicles with the appropriate target membrane. Only if the SNARE proteins on both target and vesicle membrane match, fusion occurs. Knockdown of two closely related VAMP72 proteins in *Medicago* inhibits the formation of a cell-wall-free interface in both the *Rhizobium* and AM symbiosis. Further, growth of the root, nodule formation, and infection thread/trunk formation are not affected. So the exocytotic pathway in which these VAMP72 SNAREs participate is essential for the formation of the symbiotic interface but not for other exocytosis-dependent processes.

So the comparison of the mechanism controlling symbiotic interface formation in AM and *Rhizobium* symbiosis strongly suggests that the signaling and cellular processes controlling symbiotic interface formation in the ancient AM symbiosis have been recruited by the *Rhizobium* nodule symbiosis. Some elements of the common signaling pathway are essential for the formation of the symbiotic interface in the *Frankia* nodule symbiosis (see Chapters 42, 43). Therefore, we hypothesize that also in this symbiosis, both signaling and cellular processes have been co-opted from the AM symbiosis.

4.8 INTERACTIONS WITH (BIOTROPHIC) PATHOGENS

In addition to endosymbiotic interactions also in interactions with biotrophic fungi, an interface needs to be created. These are formed around the intracellular feeding structures developed by the fungus to feed on the plant (Fig. 4.1d). In these biotrophic interactions, the fungal hyphae first enter the plant intercellularly. Inside the root, the hyphae branch and penetrate host cells to form haustoria. These haustoria are surrounded by an extrahaustorial membrane, a host membrane that is connected to the plasma membrane. However, a cell wall is lacking (Ivanov et al., 2010). So this interface is similar to the symbiotic interface of AM fungi, *Frankia* and *Rhizobium* in *Parasponia* and primitive legumes. This raises the question whether the biotrophic pathogenic and symbiotic interactions of fungi and plants are evolutionary related, and if so which is the oldest. Some studies showed that the common signaling pathway is not required for haustorium formation. However, this is especially involved in pathogens that interact with leaves (Mellersh and Parniske, 2006). For the

genes encoding components of the common signaling pathway, it is known that they are hardly expressed in the shoot (Benedito et al., 2008). Therefore, it is not so strange that they are not involved in the interaction with pathogens in leaves. It remains to be studied whether root biotrophs use the common signaling pathway and which exocytotic pathway is involved.

In biotrophic interactions, plants form an interface and are "forced" to support the pathogen. However, a more general response to pathogens is defense. Plants recognize the pathogens by the so-called PAMPs (pathogen-associated molecular patterns). One of the PAMPs that plants can recognize is chitin. This chitin forms the backbone of both the Nod factor and the Myc factors (LCOs and short chitin oligomers). The similarities in the structure of these signaling molecules indicate that the perception of chitin is also similar to the perception of Nod and Myc factors. Therefore, the recognition of these symbiotic signals may originate from pathogenic interactions or vice versa.

In rice, chitin oligomers are recognized by two receptors: CEBIP and CERK1. CEBIP has several extracellular LysM domains, but lacks an active kinase. CERK1 is an LysM receptor-like kinase. This is very similar to the Nod-factor receptors (Fig. 4.2) (Gough and Cullimore, 2011; Nakagawa et al., 2011; see Chapter 51). When the extracellular part of the Nod factor receptor is combined with the intracellular part of CERK1, this chimeric receptor is able to function as a Nod-factor receptor. This shows that the intracellular part of the receptor hardly changed.

Furthermore, it is shown that in *Lotus*, that Nod factor not only induces symbiosis but also activates defense-related genes (Nakagawa et al., 2011). When the receptors are expressed in *Nicotianum* leaves, they cause cell death in the absence of Nod factors (Madsen et al., 2011; Pietraszewska-Bogiel et al., 2013). These studies underline the close relationship of these pathogenic and symbiotic receptors.

In *Parasponia*, it is shown that the kinase death NFP receptor is required for Nod factor as well as for mycorrhizal responses. However, whether a second LysM domain receptor-like kinase is involved in the AM symbiosis is not known. Information about the LysM domain receptors involved in the AM symbiosis will be important to resolve the evolutionary relationship of these symbiotic and pathogenic receptors.

4.9 THE EVOLUTION OF THE *RHIZOBIUM* NODULE SYMBIOSIS

Studies that are described before strongly indicate that the *Rhizobium* nodule symbiosis evolved from the more ancient AM symbiosis. In both cases, a symbiotic interface forms the heart of the symbiosis as it allows an intracellular hosting of the microsymbiont and a well-controlled exchange of nutrients. The cellular mechanism by which the interface is formed involves the same exocytotic pathway (Ivanov et al., 2012). Also the signaling mechanism by which responses are induced in these two endosymbioses is, in part, still identical (the common symbiotic pathway). Some elements, for example, the LCO (Nod factor) receptors, have probably diverged due to gene duplication and neofunctionalization. This hypothesis is especially based on studies in *Parasponia*, which revealed that the same receptor is essential in both interactions and identification of the structure of LCOs made by AM fungi. The latter revealed that AM LCOs have a very similar structure as basal Nod factors (see Chapter 51). However, as rhizobia can produce decorated LCOs that play an important role in host specificity, co-evolution of receptors and Nod factor structure should have taken place.

The *Rhizobium–Parasponia* symbiosis evolved independently from the legume–*Rhizobium* symbiosis. The fact that both are based on Nod factor induced signaling points to an important evolutionary constraint, namely that the AM mechanisms form the foundation for the evolution of the *Rhizobium* symbiosis. Phylogenetic data suggest that in the Fabaceae, the symbiosis has evolved up to six times. It will be interesting to determine whether in all cases the AM machinery was the evolutionary driving force (Doyle, 2011; Geurts et al., 2012; Streng et al., 2011). Further support for the importance of the AM symbiosis for the

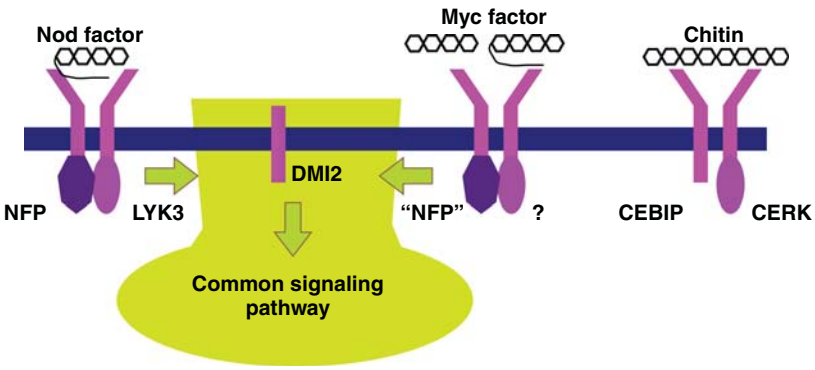

Figure 4.2 Perception of symbiotic and pathogenic signals is similar. Two LysM-type receptors perceive the signal of which one has no (active) kinase. The signal is a chitin-derived molecule. For the perception of the Myc factor in legumes NFP is not needed, but in the nonlegume *Parasponia* it is essential. Likely, in legumes another receptor performs this function.

evolution of N-fixing nodule symbiosis comes from the actinorhizal–*Frankia* symbiosis. This interaction has been shown to depend on the homolog of SymRK from the common signaling pathway (see Chapters 42, 43). Therefore, we also propose that this endosymbiosis evolved from the AM symbiosis.

The AM symbiosis is maintained in the vast majority of land plants. Therefore, the observation that this symbiosis forms the evolutionary blueprint for the N-fixing endosymbioses shows that these novel symbioses evolved by co-opting rather common mechanisms. These N-fixing nodule symbioses further evolved by recruiting other common processes. Examples are the cell cycle machinery that is modified to support infection thread growth (Yang et al., 1994) and recruitment of the transcriptional regulators of strigolactone biosynthesis as the key regulator of nodule symbiosis (Liu et al., 2011).

The co-option of common processes during nodule evolution seems to contrast with earlier studies on molecular mechanisms, controlling root nodule formation. Before the development of model legume systems that allowed the cloning of mutated genes, studies were especially focused on genes that were particularly expressed in root nodules (nodulins) (Bisseling et al., 1983; Legocki and Verma, 1980). These studies were, in part, driven by the idea that the evolution of nodule symbiosis had created novel genes. However, further studies on nodulin genes revealed that they are often expressed in other organs, albeit at a low level. Furthermore, several nodulins are the result of gene duplication by which nodule-specific variants could evolve. Examples are Flotillins (Haney and Long, 2010) and Remorins (Lefebvre et al., 2010; Toth et al., 2012). The importance of gene duplication in the refinement of the nodule symbiosis is also shown by the analysis of the *Medicago* genome (Young et al., 2011). This revealed that a whole genome duplication contributed most likely to the evolution of nodulation. Of the whole genome duplication, a certain percentage of the genes is maintained. From these maintained genes, only a subset is expressed during nodulation. The notion that nodulins are late additions in nodule evolution is supported by the fact that many of these genes are specific to the *Rhizobium*–legume symbiosis and not shared with the AM symbiosis (Deguchi et al., 2007; Manthey et al., 2004).

Although there are many similarities between the *Rhizobium* and AM symbiosis, there must be some striking differences, otherwise all plants would be able to establish a *Rhizobium* symbiosis. A major difference between the two symbioses is the fact that rhizobia never establish a symbiotic interface in existing/normal root cells, where AM fungi always do. AM fungi form arbuscules in root cortical cells, whereas rhizobia always form an interface in newly formed cells, mostly in root nodules. Even a basal legume such as *Gleditsia triacanthos* that does not form root nodules induce cell divisions in the root cortex to create an environment in which the bacteria can be hosted intracellularly (Fehér and Bokor, 1926). We hypothesize that in normal root cells the turgor pressure is too high to allow the release of symbiosomes from an infection thread. In contrast, a fungus can create force to enter a plant cell against the turgor of the host cell allowing intracellular growth (Howard et al., 1991). Therefore, we hypothesize that a major step in the evolution of the *Rhizobium* symbiosis is the formation of a cell type, probably with a temporal reduced turgor pressure, that allows the formation of a symbiotic interface by bacteria.

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Chapter 5

Bioengineering Nitrogen Acquisition in Rice: Promises for Global Food Security

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5.1 INTRODUCTION

Nitrogen (N) is the most often limiting of the nutrients that terrestrial plants need to acquire from the soil (Reich et al., 2006; Tilman et al., 1996; Vitousek and Howarth, 1991; Vitousek et al., 2010). On account of this, N additions in the form of fertilizers, largely derived from the Haber–Bosch process, to agricultural fields have produced among the largest successes in boosting crop growth and yield in the past 100 years (Tonitto et al., 2006). Currently, some 80–170 Tg of N are applied to crops annually (Cassman et al., 2002; Smil, 1999), representing a more than 8.6-fold increase in the past 40 years alone (Mulvaney et al., 2009). In the world's number one crop species, rice, N applications are particularly dramatic, and most modern varieties require very high input rates, as high as 7×10^6 tons of elemental N per year (Cassman et al., 1993, 1998; Dobermann and Cassman, 2002; Khush, 1995; Kropff et al., 1993; Matson et al., 1997; Sheehy et al., 1998). To further aggravate the problem, fertilizer-use efficiency for N is low in practical settings, and in most cropping systems some 45–55% of applied N is lost back to the environment through denitrification, volatilization, leaching, and erosion (Galloway and Cowling, 2002; Smil, 1999; Tonitto et al., 2006). In rice, the typical recovery rate for N applied through fertilizers in aboveground biomass is even lower and typically does not exceed a third (Cassman et al., 1993, 1998, 2002; Hakeem et al., 2011; Kirk and Kronzucker, 2005; Kronzucker et al., 2000; Li et al., 2012b; Vlek and Byrnes, 1986). As a result, the need for improving N-use efficiency in rice is a major priority, and has, in recent years, increasingly guided molecular biology approaches that are hoped to lead to new, bioengineered, rice lines with optimized N acquisition traits in the near future. Some of these approaches, early successes, associated challenges, and hopeful developments are reviewed here.

5.2 PRIMARY NITROGEN UPTAKE AND NITROGEN-USE EFFICIENCY: GENE CANDIDATES AND CAVEATS

The main form of N available in most rice soils, in particular the irrigated paddy soils in which the bulk of the world's rice is grown, is that of ammonium-N (NH_4^+) (Arth et al., 1998; Kirk and Kronzucker, 2005; Shen, 1969; Shrawat et al., 2008; Tabuchi et al., 2007; Wang et al., 1993a, b). Meanwhile, the NH_4^+-use efficiency of rice in field settings is exceptionally low (Cassman et al., 2002; Li et al. 2012c; Rehman et al., 2013). Biotechnologists have, thus, shown great interest in the primary process of NH_4^+ uptake by the rice plant (Britto and Kronzucker, 2004). At the physiological level, NH_4^+ uptake in rice is well characterized (Bonner, 1946; Fried et al., 1965; Kronzucker et al., 1998, 1999, 2000; Shen, 1969; Wang et al., 1993a, b, 1994), and the main molecular players have been identified. Of the transport systems situated in root plasma

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membranes responsible for catalyzing NH_4^+ uptake, the best characterized are the members of the AMT family of transporters. Indeed, a member of the AMT family was the first high-affinity NH_4^+ transporter identified in higher plants, and AMT transporters are now well studied at the molecular level in *Arabidopsis thaliana* (Gazzarrini et al., 1999; Loqué and von Wiren, 2004; Ninnemann et al., 1994; Sohlenkamp et al., 2000, 2002), tomato (Lauter et al., 1996; von Wiren et al., 2000), *Lotus japonicus* (D'Apuzzo et al., 2004; Salvemini et al., 2001; Simon-Rosin et al., 2003), *Brassica napus* (Pearson et al., 2002), and rice. Localization of the transporter proteins in plasma membranes is now solidly established (Ludewig et al. 2002, 2003; Loqué et al. 2006; Simon-Rosin et al. 2003; Sohlenkamp et al. 2002; Yuan et al. 2007a). In the *Arabidopsis* model system, mutant analysis has allowed the conclusion that three members of the AMT1 subgroup of the transporter family, specifically AtAMT1;1, AtAMT1;3, and AMT1;5, account for as much as 80% of total root uptake in that species (Loqué et al., 2006; Yuan et al., 2007a, b). Recent studies have shown that a minimum of 12 members of the gene family are present in rice, and that these differ greatly in tissue distribution and expression response to NH_4^+ withdrawal and resupply (Gaur et al. 2012; Li and Shi, 2006; Li et al., 2009a, 2012b; Kumar et al., 2003, 2006; Sonoda et al., 2003a, b; Suenaga et al., 2003; Yao et al., 2008). At this juncture, it is critical to emphasize that most NH_4^+ transporters, and the transport functions catalyzed by them, are subject to potent feedback cycles, that is, display upregulation in response to NH_4^+ withdrawal and downregulation in response to NH_4^+ resupply, processes that are affected both by NH_4^+ itself and by amino compounds, such as glutamine, that are synthesized in NH_4^+ metabolism (Kronzucker et al., 1996, 1998; Rawat et al., 1999; Sonoda et al., 2003a, b; Wang et al., 1993a, b). This reality is summarized in Figure 5.1. The inset in Figure 5.1 illustrates the rapidity of these up- and downregulation responses; peaks in transport function are achieved within 48 h in rice (Li et al., 2012b) or even faster (Kronzucker et al., 1998; Wang et al., 1993a), and responses in transcript abundance for some members of the *AMT* family are already detectable within 30 min (Sonoda et al., 2003b; Tabuchi et al., 2007).

It is an often neglected reality that these plasticities in expression and function in response to nutritional "filling state" must, by their very nature, present a formidable obstacle to improving NUE from the perspective of simple overexpression of transporters, as such overexpression is bound to result in even more rapid downregulation of transport at both transcriptional and post-transcriptional levels than would occur under "natural" conditions (see Britto and Kronzucker, 2004, 2005, for an extensive discussion of this constraint). Even more importantly, toxicity can, in fact, result from simple overexpression, which has been observed for *OsAMT1;1* (Hoque et al., 2006). Such toxic responses likely occur because of unrestrained NH_4^+ uptake and tissue

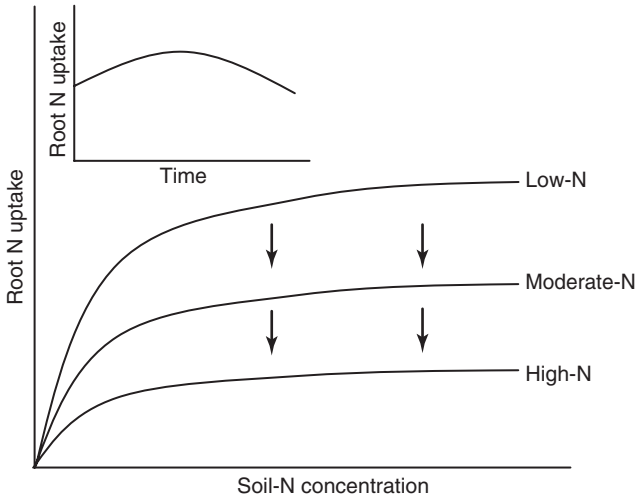

Figure 5.1 Feedback regulation of NH_4^+ influx in rice, as influenced by the level of N supply during growth (main panel; based on Wang et al., 1993b), and in response to N withdrawal for an extended period (here: 96 h) followed by NH_4^+ resupply (inset; based on Li et al., 2012b). Arrows indicate downregulation of fluxes with increasing steady-state N status. Data based on labeling experiments with the radiotracer ^{13}N (main panel) and the stable tracer ^{15}N (inset).

accumulation of the ion (Balkos et al., 2010; Britto et al., 2001; Britto and Kronzucker, 2002; Chen et al., 2013; Li et al., 2012a). Of the large number of rice AMT transporters, only three *OsAMT1* members, and *OsAMT2;1*, have thus far been examined in heterologous yeast expression systems, clearly exhibiting NH_4^+-transport activity (Sonoda et al., 2003a, b; Suenaga et al., 2003), while *in planta* analyses have remained scarce (Kumar et al., 2003, 2006; Li et al., 2012b). However, a recent detailed analysis of expression responses of *OsAMTs* (Li et al., 2012b), in conjunction with earlier studies (Sonoda et al., 2003a, b), has shed light on the variable responses of the key members in the rice system. In Table 5.1, we summarize the chief tissue/organ localization (root vs shoot) of these members of the transporter family, and their responses to NH_4^+ withdrawal and resupply. It emerges from this analysis that the main players for NH_4^+ uptake by the rice root system are *OsAMT1;1*, *OsAMT1;2*, and *OsAMT1;3*, of which the first is also expressed in shoots (and is both constitutive and inducible; Tabuchi et al., 2007), but the latter two are predominantly present in the roots, whereas other members are characterized by predominant shoot expression (Li et al., 2012b). Of these, *OsAMT1;1* and *OsAMT1;3* show clear inducibility, that is upregulation of transport in response to N withdrawal (Table 5.1), in keeping with classic expectation (Fig. 5.1). Data on *OsAMT1;3* are conflicting at present, both in the sense that downregulation has been recorded in response to N withdrawal (Li et al., 2012b) and resupply (Sonoda et al., 2003b), and in that

Table 5.1 The main NH_4^+ transporters in rice, their expression in root versus shoot, and the response to N withdrawal and resupply (based on Li et al., 2012b)

Ammonium Transporter	Root Expression	Shoot Expression	Response to NH_4^+ Withdrawal	Response to NH_4^+ Resupply
OsAMT 1;1	✓	✓	↑	--- (↑)
OsAMT 1;2	✓	Minor	↑	↓
OsAMT 1;3	✓	Minor	↓	↑
OsAMT 2;1	✓	✓	↑	--- (↑)
OsAMT 2;2	Minor	✓	↑	↑
OsAMT 2;3	✓	✓	↓	↓
OsAMT 3;1	Minor	✓	↑	↑
OsAMT 3;2	Minor	✓	↑	↑
OsAMT 3;3	Minor	✓	↑	↓

Check marks indicate the predominant sites of expression, and Minor marks a minor presence. Up and down arrows indicate whether a gene is up- or downregulated under either NH_4^+ withdrawal or resupply, respectively (strong arrows indicate strong changes, small arrows small changes; dashed lines indicate little to no change). Bold indicates genes that show the highest promise for enhancing NUE in roots (for important caveats, however, see text).

resupply has also been shown to increase expression in some cases (Li et al., 2012b).

Importantly, expression levels differ at different stages of development of the rice plant, and, for instance, *OsAMT1;2* and *OsAMT3;1*, are upregulated significantly in the critical tillering stages in roots and shoots, respectively, while playing relatively minor roles in earlier vegetative stages (Li et al., 2012b). One may deduce from this survey that a most promising route for the biotechnologist would be to target the expression of *OsAMT1;1* and *OsAMT1;2* to higher levels than in the native state, if the goal is to increase agronomic NUE from the perspective of primary N uptake by rice roots, with special emphasis on *OsAMT1;2* given its importance in the stages of tillering. However, we underscore and extend our caveat expressed previously (see also earlier) pertaining to such attempts, on account of the native dynamics of downregulation (following the principle: "what goes up must come down"), summarized conceptually in Figure 5.1, and the possibility of excess tissue NH_4^+ buildup, which is known to carry toxic consequences (Barker 1999; Li et al., 2012a; You and Barker 2004), even in rice (Balkos et al., 2010). Altogether these realities create a forbidding scenario in the pursuit of this goal and explain the limited success to date (Hoque et al., 2006).

Some reports are also available in the literature that suggest that NH_4^+ may be taken up by some plants and, under some circumstances, by other transporters, such as nonselective cation channels (Demidchik et al., 2002; White, 1999), potassium channels (Bertl et al., 1997; ten Hoopen et al., 2010; see also Kronzucker and Britto, 2011), or aquaporins (Jahn et al., 2004). Although most do not consider these pathways to be significant under normal agronomic conditions (Balkos et al., 2010), they may yet offer potential as alternative targets for improving NUE but might also pose problems when attempting to increase NUE by targeting of AMT transporters, as such parallel pathways, where present, may compensate in unpredictable ways.

We should also add a comment on nitrate-N: it is important to keep in mind that, while the bulk of N present in the most intensely cultivated rice soils is in the NH_4^+ form, there is some presence, and production, of NO_3^-, in particular in the rhizospheric microenvironment that experiences oxygen extrusion into the rooting medium via aerenchyma tissue in the rice plant (Kirk and Kronzucker, 2005; Li et al., 2008). This production of NO_3^- can be facilitated by periodic wetting–drying regimes in rice paddies, a process that fosters nitrification in the soil (Kirk and Kronzucker, 2005; Kronzucker et al., 2000), and indeed, under some conditions, as much as 40% of the total N in the rice plant may derive from the NO_3^- source (Kirk and Kronzucker, 2005). Furthermore, the co-presence of NO_3^- along with NH_4^+ has been shown to produce significant synergistic growth responses in rice, as it does in many other species (Kronzucker et al., 1999). Thus, an examination of NO_3^- transporters in addition to those that mediate NH_4^+ uptake may prove useful in the future. Recent studies have examined the expression responses of the chief members responsible for NO_3^- uptake in rice from agronomically relevant concentrations (Araki and Hasegawa, 2006; Tang et al., 2012; Yan et al., 2011), members of the *OsNRT2* group of genes (Plett et al., 2010), and using knockdown approaches for transporters involved in the long-distance nitrate transport from root to shoot, Tang et al. (2012) showed that, in the NO_3^- case, there may yet exist possibilities of uncoupling from negative-feedback loops of the type displayed in Figure 5.1 for NH_4^+ uptake. This, in turn, holds promise for increasing NUE that deserves further exploration. Also interesting in this regard is the observation

that negative feedback in rice appears to proceed differently than in the model system *Arabidopsis* (Tang et al., 2012), underscoring the importance of studying the rice system directly, rather than relying on models that derive only from the study of the *Arabidopsis* system.

5.3 THE CARBON–NITROGEN INTERFACE AND N-TRANSFER: REMOVING ENZYMATIC BOTTLENECKS

Once N is taken up by the plant, it must be assimilated to drive growth, and it is at this juncture of carbon and nitrogen metabolism where promise for improvement of NUE in the rice plant might be especially high (Britto and Kronzucker, 2004; McAllister et al., 2012; Shrawat et al., 2008; Tabuchi et al., 2007). For the NO_3^- source of N, attempts on increasing NUE by virtue of reengineering the entry step of NO_3^- into metabolism, that of nitrate reductase (NR), have been largely disappointing (Crawford, 1995; Britto and Kronzucker, 2004), and, although patent applications based on NR genes from algae supporting the approach have been filed (Loussaert et al., 2010; Liu et al., 2011; see also McAllister et al., 2012), the promise of this target point is not generally considered high (see Britto and Kronzucker, 2004, 2005, for more discussion). For the entry step of NH_4^+ into metabolism, however, that is, the incorporation of N into carbon skeletons via glutamine synthetase (GS), more potential is typically assumed. As the importance of NO_3^- for rice, while not negligible (see earlier), is clearly secondary to that of NH_4^+, this potential is of particular interest in this discussion. Early successes based on manipulation of GS include biomass stimulation in *Lotus corniculatus* (Vincent et al., 1997) and the stimulation of height growth in trees, which was recorded to reach 76% in initial stages of growth, although this gain declined to only ~21% in later stages (Gallardo et al., 1999). Biomass increases, based on both overexpression of the genes coding for the various forms of the GS1 isoform (rice has three *GS1* gene family members alone; Tabuchi et al., 2007) and those for GS2 (this isoform is localized predominantly in chloroplasts, where it is linked to the photorespiratory N cycle; Husted et al., 2002), have been reported in *L. japonicus* (Hirel et al., 1997) and tobacco (Fuentes et al., 2001; Migge et al., 2000; Oliveira et al., 2002). Results on rice have been more difficult to evaluate, even though some positive results have been obtained, albeit not in relation to growth and yield under "normal" conditions without superimposition of stress (Britto and Kronzucker, 2004; Hanzawa et al., 2002; Hoshida et al., 2000; Tabuchi et al., 2007). One of these reports has shown enhanced stress tolerance by virtue of GS2 overexpression, however, in particular in the context of salt stress (Hoshida et al., 2000), which may be attributed to enhanced substrate flow through photorespiration (Kozaki and Takeba, 1996). A more recent appraisal of successes in improving NUE in rice via GS manipulation has been more critical, however (Brauer et al., 2011). Similarly, a positive report on account of overexpression of another key enzyme of primary N assimilation, glutamate dehydrogenase (NADH-GOGAT) in tobacco (Ameziane et al., 2000) has, unfortunately, not as yet been corroborated in large-scale studies or in cereals, even though the enzyme's role in primary N metabolism and remobilization, and its tissue distribution, are well established in rice (Tabuchi et al., 2007).

One particularly exciting development in modifying internal N metabolism has been the targeting (using tissue-specific expression, an approach of increasing promise in genetic engineering; see Møller et al., 2009) of a gene coding for an aminotransferase enzyme, alanine aminotransferase (*AlaAT*; Good et al., 2007; McAllister et al., 2012, 2013; Shrawat et al., 2008). It has been argued that the gene and its product may also serve as a useful marker for NUE in general (Cañas et al., 2010). Most importantly, the role of *AlaAT* expression has been examined in rice. Table 5.2 summarizes the increases in root, shoot and total biomass, N content, and seed yield obtained in overexpressor lines of *Japonica* rice (Shrawat et al., 2008). The gains reported are impressive and are easily in line with the best results achieved via GS manipulation in the most optimistic settings (see earlier and Britto and Kronzucker, 2004). In general, the potential for alterations in internal N cycling, in particular in relation to N remobilization from previously synthesized organic N compounds in aboveground tissue, including during the important process of tiller formation and grain filling, appears significant (El-kereamy et al., 2012; Fei et al.,

Table 5.2 Increases in biomass, total N, and seed yield in roots, shoots, and whole plants, on a dry-weight basis of overexpressor lines for the gene-encoding alanine aminotransferase (*AlaAT*) in 45-day-old *Japonica* rice (cv Nipponbare; after Shrawat et al., 2008)

	Root Tissue	Shoot Tissue	Total Plant at Maturity
Total biomass, %	+9–51	+26–47	+30–34
Total N, %	+14–54	+40–65	**+36–61**
Seed yield, %	N/A	N/A	**+31–54**

2003; Good et al., 2004; Seger et al., 2009), and may present a promising alternative to modifications of primary-uptake mechanisms.

One area that is peripherally related to N acquisition and metabolism where success has been achieved, and more may be expected in the future, is in the targeting of an enzyme that plays an especially important role in C_4 species, phosphoenolpyruvate carboxylase, PEPC (Britto and Kronzucker, 2004). PEPC is a key enzyme in C_4 plants, where it is responsible for primary C fixation, and has been engineered into an overexpressed state into rice, using a *PEPC* gene from corn, with yield data indicating a 10–30% increase (Jiao et al., 2002). This success was even more impressive when a second enzyme, PPDK, was co-expressed with PEPC – additional 5–10% increases were seen in this approach. This work is interesting in that it indicates that the feasibility of achieving a partial C_4 habit in a major C_3 crop (Britto and Kronzucker, 2004), and engineering the superior carbon- and water-use efficiencies characteristic of C_4 species into rice is a major goal in modern rice research (Sage and Zhu, 2011). We have previously argued that optimizing the PEPC apparatus in rice may also be critical because of improved (anapleurotic) carbon supply for N metabolism, and we have also shown that such a feat may be accomplished by nutritional poising of nutrient media, in particular through the optimization of supply ratios of K^+ to NH_4^+ (Balkos et al., 2010; see also Roosta and Schjoerring, 2008).

5.4 NITROGEN FIXATION: THE HOLY GRAIL

One of the most elusive, and yet also most promising, targets pursued by plant biologists with respect to increasing NUE in rice has been achieving biological nitrogen fixation (BNF) within the rice plant, much like members of the Leguminosae family (Beatty and Good, 2011; Britto and Kronzucker, 2004; Ladha and Reddy, 2003; Shantharam and Mattoo, 1997). Such an achievement could relieve the enormous economic and environmental burden presented by fertilizer application, which costs upward of $100 billion per year and causes alarming environmental N pollution (Beatty and Good, 2011; Britto and Kronzucker, 2002; Galloway et al., 2008; Vitousek et al., 2009). Although this continues to be a difficult long-term prospect, especially considering the complex molecularly machinery involved (Charpentier and Oldroyd, 2010), its success in crops like rice is not considered insurmountable (Britto and Kronzucker, 2004; Beatty and Good, 2011). As discussions of details of N fixation fill the pages of this book, we will limit ourselves to the broader strokes pertaining to the rice system in particular.

One intensely pursued strategy has involved developing root nodule symbioses with diazotrophic bacteria (N-fixing bacteria containing the nitrogenase enzyme system) similar to those found in legumes (de Bruijn et al., 1995; Khush and Bennett, 1992; Ladha and Reddy, 1995; Oldroyd et al., 2009). The key to achieving such a goal involves engineering bacteria to first recognize and infect the host cells of the rice root system, followed by engineering of nodule organogenesis in the rice plant itself, so as to encapsulate bacteria in a low-oxygen environment. The latter is critical to support the bacteria's oxygen-sensitive nitrogenase enzyme complex (Gallon, 1981; see Chapter 108). In legumes, a complex signaling crosstalk between rhizobia and the plant initiates nodulation, whereby the secretion of phenolic compounds (particularly flavonoids) from the plant triggers bacteria to secrete nodulation (Nod) factors that promote nodule formation in the plant (Denarie and Cullimore, 1993; see Chapter 51). Interestingly, rice appears capable of perceiving Nod factors, and several homologs to legume "early nodulin" genes (ENODs) have been found in rice (Reddy et al., 1999, 2000). A possible explanation to this may be found in the similarities, both at the genetic (Hirsch and Kapulnik, 1998; Venkateshwaran et al., 2013; see also Chapter 108 by de Bruijn in this book) and functional (Maillet et al., 2011) levels, between plant symbioses with diazotrophs and those with arbuscular mycorrhizal (AM) endosymbiotic fungi, the latter of which are found in 70–90% of terrestrial plants, including rice (Khan and Belik, 1995). Thus, exploiting the endogenous AM (Myc-based) signaling pathway in rice to engineer diazotroph symbioses appears to be the most promising avenue. Further engineering progress is likely to involve bacterial entry and nodule organogenesis – two processes for which our understanding is still poor, although is quickly advancing (Charpentier and Oldroyd, 2010). Initial indications are that similar mechanisms exist between AM-fungal and rhizobial invasions of plant roots, which bode well for establishing BNF in rice (Madsen et al., 2010; see Chapter 108). Moreover, closely related research into the development of symbiotic nitrogen fixation in maize, based on "common pathway genes" among legumes and monocots, has recently received considerable financial support from the Bill and Melinda Gates Foundation (www.foundationcenter.org/gfp/foodsecurity; see also Chapter 109 by Beatty et al. in this book). While no true nodule formation has been demonstrated in rice thus far, early indications are that nodule formation in legumes uses plant hormones that are in fact ubiquitous throughout the plant kingdom rather than being unique to legumes (Madsen et al., 2010). Also, earlier studies have demonstrated morphological responses to rhizobial inoculation in rice, such as short and thick roots (Reddy et al., 1997) and root hair deformation, an early response involved in bacterial infection (Reddy et al., 2000). However, a key understanding that is currently lacking is what produces the specificity of responses in legumes to changes in auxin and cytokinin levels that initiate nodulation-specific developmental processes.

Indeed, it has long been known that rice already takes advantage of BNF under natural conditions, where N-fixing endophytic rhizobia form nodule-independent associations with rice (Ladha and Reddy, 2003; Stoltzfus et al., 1997; Yanni et al., 2001; see Chapter 88). Although the addition of some of these bacteria to fertilizer mixtures has yielded positive results in rice (e.g., ~30% increase in yield and agronomic NUE; Yanni et al., 2001; see Chapter 111), it remains contentious whether yield increases are actually due to BNF. One field study indeed attributed yield increases with biofertilizers not to BNF, but to changes in root morphology, stimulated by rhizobial production of the plant growth hormones IAA and GA7, which appeared to improve soil extraction of nutrients including N (Yanni et al., 2001). Although new rice endophytes that fix nitrogen at higher rates are currently being screened (Yanni and Dazzo, 2010), it would appear that such a strategy is limited in terms of the amount of fixed nitrogen that can be achieved.

Perhaps the most challenging strategy for BNF in rice is to directly introduce the nitrogenase enzyme system into a plant organelle so that the plant can fix its own nitrogen (Merrick and Dixon, 1984; see Chapter 108). Such a task may require incorporating the 16 nitrogen fixation (*nif*) genes essential to nitrogenase activity into the rice genome (Ladha and Reddy, 2003). The products of at least six genes, *NifB*, *NifEN*, *NifH*, and *NifDK*, have been shown to be absolutely required for FeMo-co biosynthesis and nitrogenase activity both *in vivo* and *in vitro*, while products of some genes that are required for FeMo-co biosynthesis could be replaced by the activities of plant counterparts (Jimenez-Vicente et al., Chapter 7; de Bruijn, Chapter 108, this book). It has been proposed that the ideal locations for introducing these genes are mitochondria and chloroplasts, rather than cell nuclei, as plastidic genetics are most similar to those of N-fixing bacteria (Whitfeld and Bottomley, 1983), and these organelles can provide high concentrations of ATP and reducing power required for nitrogenase activity (Merrick and Dixon, 1984). A potential problem with this approach, however, lies in the oxygen sensitivity of nitrogenase (see earlier), as virtually all plant cells process or generate oxygen, particularly chloroplasts. This could theoretically be overcome by regulating *nif* expression in chloroplasts diurnally, such that nitrogen fixation only occurs at night. Also, oxygen-sensitive enzymes within this organelle are not without precedent (Muraki et al., 2010). Conversely, the issue of oxygen sensitivity could be circumvented by limiting *nif* expression to root plastids in rice, where photosynthetically produced oxygen is absent. Mitochondria also have efficient oxygen-consuming respiratory enzymes and functioning oxygen-sensitive enzymes (Lill and Mühlenhoff, 2008); thus, they are also suitable candidates. Yet another potential solution involves the expression of the oxygen-tolerant nitrogenase found in the bacterium *Streptomyces thermoautotrophicus* (Ribbe et al., 1997). While such strategies are, in principle, achievable, they will likely require many more years of intensive research and development.

5.5 CONCLUDING REMARKS

We show here that many attempts to improve NUE in rice have been contemplated and attempted, but a large number of these have been disappointing (Good et al., 2004), although new funding from the Bill and Melinda Gates Foundation has newly stimulated this research (see earlier). It now emerges that, in addition to focusing on the primary intake step of N in the quest to improve NUE in rice, targeting downstream steps in metabolism may hold considerable promise. This is illustrated in successes such as those associated with the overexpression of the gene coding for alanine aminotransferase (Shrawat et al., 2008). We believe successes in this area may be related to the potential for relief from negative feedback of N transport (Fig. 5.1) that is exerted both by the externally provided N sources (NH_4^+ and NO_3^-) themselves and by endogenous N compounds formed in the N-metabolic machinery. Any approach, therefore, that may facilitate at least partial uncoupling from the potent downregulatory effects of many of these compounds by changing the internal chemical N signature may "bring out" the potential for uptake that is already in place without the need for insertion of foreign transporter genes. In the case of K^+ uptake, we have recently shown how pronounced the inherent plasticity of transport may be, based on the already present set of transporter genes, and how simple alterations of the nutrient environment may unlock this potential, resulting in substantial benefits to tissue nutrient accrual and growth (Coskun et al., 2013; Coskun and Kronzucker, 2013). Similarly, recent studies in the "nutritional poising" of NH_4^+ uptake and metabolism by potassium (Balkos et al., 2010; Roosta and Schjoerring, 2008) and the well-established growth and yield synergism on mixed N sources (Kirk and Kronzucker, 2005; Kronzucker et al., 1999) illustrate that a similar potential exists in the rice plant with respect to N acquisition, that is, successes in improving NUE in the field may not only come about through biotechnological manipulations of the genome, but may also include nutritional modifications of the rooting environment, which may be achieved through agricultural management practices such as imposition of wetting/drying regimes in paddy fields or precision farming (Cassman, 1999; Balasubramanian et al., 2004). In addition, the area of achieving N-fixation symbiosis in the rice plant remains a "holy grail" for plant biologists, and research in this area is benefitting from an upswing in activities, in part, driven by new funding (see earlier; see Chapters 108, 109). The improvement of NUE in rice, by any number of means, remains a very high priority, given the enormous challenge

of assuring global food security under currently flattening per capita yield increases, and the premier position rice holds, and will continue to hold, as a staple food for nearly half the human race.

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Section 2

Chemistry and Biochemistry of Nitrogenases

Chapter 6

An Overview on Fe–S Protein Biogenesis from Prokaryotes to Eukaryotes

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6.1 INTRODUCTION

Iron–sulfur (Fe–S) proteins are ubiquitous and evolutionary ancient prosthetic groups that are essential in maintaining basic processes of life such as photosynthesis, respiration, central metabolism, cofactor biosynthesis, ribosome biogenesis, gene regulation, RNA modification, replication, nitrogen fixation (see Chapter 2), and DNA repair (Balk and Pilon, 2011; Kesawat et al., 2012). A few billion years ago, the earth's atmosphere was plummeting into an anaerobic environment in which the earliest forms of life evolved. The abundance of iron and sulfur on earth, and possibly the structural versatility and chemical reactivity of Fe–S clusters, allowed them to gain diverse functions that are required for the fundamental processes of life. Many of the biochemical pathways that developed in this early phase of life became and remain essential to all kingdoms of life (Huber et al., 2003). Therefore, it would come as no surprise that proteins containing Fe–S clusters exist in all living organisms and play an essential role in diverse biological processes at the cellular level. Fe–S cluster formation attracted the attention of chemists and biochemists around the world in the late 1960s, when electron paramagnetic resonance spectroscopy, chemical analyses, and biophysical technique provided plenty of evidence for the presence of these versatile inorganic cofactors in a number of proteins. The spinach ferredoxin was one of the first Fe–S proteins identified and described (Hall et al., 1966; Palmer and Sands, 1966).

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It was thought for a long time that the assembly of Fe–S proteins occurred spontaneously, since the process was easily replicated chemically *in vitro* and led to the view that these cofactors can assemble spontaneously in proteins. However, genetic, biochemical, molecular, and cell biology studies in the late 1990s provided ample evidence that demonstrated that the assembly of Fe–S clusters *in vivo* is a catalyzed process rather than a spontaneous one and that it requires a plethora of genes assisting in the maturation of Fe–S clusters and their insertion into the apoproteins. Therefore, the formation of intracellular Fe–S clusters does not occur spontaneously, but requires a complex biosynthetic machinery. This was the starting point for the rapid discovery of numerous biogenesis components that participate in Fe–S cluster biosynthesis in both prokaryotes and eukaryotes (Johnson et al., 2005). Despite the relative simplicity of Fe–S clusters in terms of structure and composition, their synthesis, assembly, and transfer to apoproteins is a highly complex and coordinated process in living cells.

Over the past decade, the mechanisms of maturation of Fe–S cofactor and insertion into apoprotein *in vivo* have become an area of intense investigation. Numerous Fe–S cluster synthesis components have been discovered that assist Fe–S protein maturation according to distinct biosynthetic principles in several model organisms (Table 6.1), including *Saccharomyces cerevisiae*, *Escherichia coli*, *Azotobacter vinelandii*, *Erwinia chrysanthemi*, *Arabidopsis thaliana* as well as *Salmonella enterica*, and humans (Balk and Lobréaux, 2005; Kessler and Papenbrock, 2005; Ayala-Castro et al., 2008; Xu and Moller, 2008; Lill and Muhlenhoff, 2005; Rouault and Tong, 2008). The genes that participate in Fe–S cluster synthesis appear to be conserved in bacteria, fungi, animals, and plants (Table 6.2). With the availability of the whole-genome sequencing database, it was revealed that the Fe–S biogenesis machinery is widespread and highly conserved from prokaryotes and eukaryotes (Balk and Lobréaux, 2005; Lill and Muhlenhoff, 2005).

The presence of proteins in various species exhibiting similarity to the known ISC and CIA components of the yeast *S. cerevisiae* was evaluated by BLAST searches (Fig. 6.2). High sequence identity or similarity is indicated by ++ and +, limited sequence similarity is depicted by ?, and no evident similarity by −. Searches were performed for *Neurospora crassa*, *Homo sapiens*, *Drosophila melanogaster*, *Oryza sativa*, *A. thaliana*, the microsporidium *Encephalitozoon cuniculi*, the apicomplexa *Cryptosporidium parvum*, and the diplomonad *Giardia intestinalis*.

Three distinct Fe–S protein biogenesis systems have been identified in prokaryotes so far: the NIF system present in nitrogen-fixing bacteria (*A. vinelandii*) is specialized in the assembly of the complex Fe–S protein nitrogenase, which is responsible for the conversion of N_2 to NH_3 in nitrogen-fixing bacteria (see Chapters 2 and 7); the ISC system is responsible for the generation of the majority of cellular Fe–S proteins and thus, might perform a general housekeeping biosynthetic function particularly under normal and low oxygen concentrations. Finally, the SUF (sulfur-mobilization) machinery was discovered as an independent assemblage that might be used preferentially under oxidative stress and iron-limiting conditions (Jacobson et al., 1989; Zheng et al., 1998; Patzer and Hantke, 1999). On the other hand, three different types of Fe–S cluster biosynthesis machineries (Fig. 6.1) have been discovered in eukaryotes. The ISC assembly machinery is required for the biogenesis of all cellular Fe–S proteins (Strain et al., 1998; Kispal et al., 1999; Schilke et al., 1999; Lill and Kispal 2000). The ISC export assembly and cytosolic iron–sulfur protein assembly (CIA) machinery is required for the formation of cytosolic and nuclear Fe–S proteins (Roy et al., 2003). Here, we briefly summarize the basic principle, steps, and all the major components and mechanisms of Fe–S protein biogenesis in prokaryotes and eukaryotes.

6.2 SOURCE OF IRON AND SULFUR

Ferredoxin and frataxin (an iron-binding protein Yfh1 in yeast and CyaY in bacteria) are believed to function as potential iron donors because they interact with the sulfur donor IscS and the scaffold protein IscU. However, detailed experimental evidence to establish the roles of these genes remains to be determined. The source of sulfur in Fe–S clusters is l-cysteine, and the action of cysteine desulfurases (Nfs1–Isd11 in yeast and NifS, IscS, or SufS in bacteria) converts the l-cysteine to l-alanine, resulting in the release of the sulfur atom. As an intermediate, a persulphide is formed on a conserved cysteine residue of the enzyme. This persulphide might be transferred to conserved cysteine residues or directly to scaffold proteins. This provides an excellent mechanism for making sulfur atoms available without releasing them in solution (Mihara and Esaki, 2002).

6.3 STRUCTURES AND PROPERTIES OF Fe–S CLUSTERS

As the name itself indicates, Fe–S clusters are composed of ferrous or ferric iron and inorganic sulfide and, in rare cases, other heavy metals such as molybdenum, vanadium, nickel, or other cofactors (Rees, 2002; see Chapter 2). Fe–S clusters were discovered in the early 1960s by purifying enzymes with characteristic electron paramagnetic resonance signals, for instance, plant and bacterial ferredoxins and the respiratory complexes I–III of bacteria and mitochondria

Table 6.1 Core Fe–S cluster synthesis components in bacteria, yeast, mammalians, rice, and arabidopsis, their function, and subcellular localization

Fe–S Biogenesis Components in Yeast	Full Name	Alternatives Name	Homologs				Function	Sub-cellular Localization
			Bacteria	Mammalian	Arabidopsis Locus	Rice Locus		
ISC assembly machinery								
Nfs1	Cysteine desulfurase	ISCS	NifS, SufS-SufE, IscS	NFS1	At5g65720 At1g08490 At4g26500	Os09g16910 Os12g18900 Os09g09790a Os03g11990b	Cysteine desulfurase, sulfur donor (Cys → Ala + S), also required for thiouridine modification of tRNA	Mitochondrial matrix, nucleus
Isd11	LYR motif containing 4	Lyrm4		ISD11	At5g61220	Os10g26640, Os08g14070	Forms complex with Nfs1, required for sulfur transfer to Isu1	Mitochondrial matrix, nucleus
Arh1	Ferredoxin reductase	AdxR (adrenodoxin reductase)		FDXR	At4g32360	Os02g17700	Ferredoxin reductase, electron transfer to Yah1 from NADH	Mitochondrial matrix, inner membrane
Yah1	Ferredoxin	Fdx, Adx (adrenodoxin)	Fdx, middle domain of NifU		At4g21090	Os09g26650	Ferredoxin, reduction of an unknown substrate, possibly S0 to S2−	Mitochondrial matrix
Yah1	Ferredoxin-like	FdxL	Fdx	FDX1L	At4g05450	Os07g01930	Electron transport	Mitochondrial matrix
Mrs3, Mrs4	Mitoferrin 1, Mitoferrin 2	Mfrn, SLC25A37 SLC25A28		MFRN1, MFRN2	At1g07030, At2g30160	Os03g18550	Iron transport	Mitochondrial inner membrane
Yfh1	Frataxin	FA, FRDA	CyaY	FXN	At4g03240	Os01g57460	Putative iron donor, iron-stimulated binding to Isu1	Mitochondrial matrix, cytosol (?)
Isu1, Isu2	Iron–sulfur cluster scaffold homolog	Isu1	NifU (*N*-terminal domain), IscU	ISCU	At4g22220 At4g04080 At3g01020	Os05g49300 Os01g47340 Os01g47340	Scaffold for initial cluster assembly, interacts with Nfs1, Yfh1, Ssq1, Jac1	Mitochondrial matrix, cytosol
Nfu1	Scaffold protein	NFU1	*N*-terminal domain of NifU, NP_312283	NFU1	At4g01940 At5g49940 At4g25910 At3g20970 At1g51390	Os03g20010 Os11g07916 Os06g47940 Os11g07916 Os12g07700 Os05g06330 Os05g06330	Unknown function, genetic interaction with Isu1 and Ssq1	Mitochondrial matrix, cytosol
Grx5	Glutaredoxin 5	GRX5	Grx	GLRX5	At5g40370 At5g63030	Os04g0508300 Os02g40500 Os04g42930	Cluster transfer	Mitochondrial matrix

(*continued*)

Table 6.1 (*Continued*)

Fe–S Biogenesis Components in Yeast	Full Name	Alternatives Name	Homologs				Function	Sub-cellular Localization
			Bacteria	Mammalian	Arabidopsis Locus	Rice Locus		
Ssq1, Ssc1	heat shock 70 kDa protein 9	HSPA9, Mortalin	HscA	GRP75	At4g37910 or At5g09590	Os02g53420a Os03g02260b Os09g31486c	Specialized Hsp70 chaperone, binds to Isu1, Jac1, transfer of Fe/S clusters to target proteins? Cluster transfer	Mitochondrial matrix, cytosol (?)
Jac1	HscB iron–sulfur cluster co-chaperone homolog	DNAJC20, HSC20	HscB	HSCB	At5g06410	Os12g27070a Os06g38950b	Cochaperone of Ssq1, targets Ssq1 to Isu1	Mitochondrial matrix
Mge1	GrpE-like1/2			GrpE-L1/2	At5g55200, At4g26780	Os02g13580 Os09g11250 Os08g25090	ADP/ATP exchange on Ssq1	Mitochondrial matrix
Isa1, Isa2	Iron–sulfur cluster assembly 1/2 homolog	Isa1, Isa2	IscA, SufA	ISCA1, ISCA2*	At1g10500 At2g16710 At2g36260 At5g03905	Os06g05400 Os12g30030 Os01g01610 Os08g28230	Biogenesis of aconitase-like Maturation of radical SAM-dependent proteins and aconitase Fe/S proteins in yeast, binds iron in yeast and bacteria, functions as alternative scaffold in bacteria	Mitochondrial matrix
Iba57	iron-sulfur cluster assembly factor for biotin synthase- and aconitase-like mitochondrial proteins with a mass of 57 kDa	Iba57		IBA57	At4g12130	Os06g0134800 Os06g04380	Maturation of radical SAM-dependent proteins and aconitase	Mitochondrial matrix
Aim1	Potential glutaredoxin-interacting protein		BolA	BOLA3	At5g09830			

Mitochondrial ISC export machinery								
Atm1	ATP-binding cassette transporter, subfamily B, member 7	ABC7		ABCB7	At4g28630	Os01g50100a Os01g74470b Os01g50080c Os01g50160d	ABC transporter, inner membrane, export of unknown compound for cytosolic and nuclear Fe/S protein maturation and iron-uptake regulation Translocation of a sulfur compound to the CIA machinery –	Mitochondrial inner membrane
Erv1	Augmenter of liver regeneration	GFER		ALR*	At1g49880	Os03g10850	Sulfhydryl oxidase in the intermembrane space, also required for protein import	Mitochondrial intermem-brane space
GSH	Glutathione			GSH			Unknown role, redox buffer in yeast	Mitochondria, cytosol
CIA machinery								
Nbp35	Nucleotide binding protein 1	NUBP1	Mrp	NBP35	At5g50960	Os02g38900a Os04g40880	Soluble P-loop NTPase, binds to Cfd1 and Nar1, can assemble additional, labile Fe/S cluster in vitro	Cytosol
Cfd1	Nucleotide binding protein 2	NUBP2	ApbC, Mrp	CFD1			Soluble P-loop NTPase, complex with Nbp35, binds labile Fe/S cluster in vitro	Cytosol
Nar1	Nuclear prelamin A recognition factor-like	PRN, HPRN, Let1L, NarfL	Iron only hydrogenase	IOP1	At4g16440	Os03g53750	Fe/S protein, binds to Nbp35 and Cia1, cluster transfer	Cytosol, nucleus
Cia1	Cytosolic iron–sulfur protein assembly 1 homolog	WDR39		CIAO1	At2g26060 At4g32990	Os07g14830 Os03g02550	WD40 domain protein, late function in biogenesis, binds to Nar1, located mainly in nucleus Docking platform, cluster transfer	Cytosol, nucleus
Dre2	Cytokine induced apoptosis inhibitor 1			CIAPIN1*	At5g18400 At5g18362	Os04g58564 Os04g57810	?	Cytosol, mitochondrial intermem-brane space (?)

Table 6.2 Conservation of the biosynthetic machineries of Fe–S protein biogenesis in various Eukaryotes including organisms lacking classical mitochondria

Protein	*N. crassa*	*H. sapiens*	*D. melanogaster*	*O. sativa*	*A. thaliana*	*E. cuniculi*	*C. parvum*	*G. intestinalis*
ISC assembly machinery								
Nfs1	++	++	++	++	++	++	++	++
Isu1	++	++	++	++	++	++	++	++
Isu2	++	++	++	++	++	++	++	++
Isa1	++	+	+	+	+	−	−	+
Yah1	++	+	+	+	+	+	+	+
Arh1	+	+	+	+	+	?	?	?
Yfh1	+	+	+	+	+	+	+	−
Nfu1	+	+	+	+	+	−	−	?
Grx5	++	+	+	+	+	+	+	+
Ssq1	++	++	++	++	++	++	++	++
Jac1	+	+	+	+	+	+	?	+
Mge1	++	+	+	+	+	−	+	?
ISC export machinery								
Atm1	++	++	++	++	++	−	?	−
Erv1	+	+	+	+	+	+	?	−
CIA machinery								
Nar1	+	+	+	+	+	+	+	+
Cfd1	+	+	+	+	+	+	+	+
Nbp35	+	+	+	+	+	+	+	+
Essential cytosolic Fe–S proteins								
Rli1	++	++	++	++	++	++	++	++

(Malkin and Rabinowitz, 1966). To date, numerous Fe–S clusters have been identified and described in virtually all organisms (Table 6.1). The most common and chemically simple forms of Fe–S clusters are the rhombic [2Fe–2S] and the cubane [4Fe–4S] types, which contain iron and sulphide. A vast majority of Fe–S clusters are usually integrated through coordination of the iron ions by cysteine or histidine residues; however, sometimes cysteine or histidine residues are replaced with aspartate, arginine, serine, peptidyl-N and non-protein ligands homocitrate, CO, and CN, particularly in more complex Fe–S clusters (Meyer, 2008). The simplest form of an Fe–S center is the rhombic [2Fe–2S] cluster, for example, Rieske Fe–S protein, ferredoxins, ferrochelatase, and biotin synthase. The [4Fe–4S] clusters can be viewed as a duplication of [2Fe–2S] clusters. This type of cluster is present in many proteins, for instance, sulfite reductase, ferredoxins, aconitase and aconitase-like proteins, and DNA glycosylase. Some proteins such as subunits of complexes I and II of the respiratory chain contain [3Fe–4S] clusters in which one corner of the cube is vacant. There are numerous proteins that contain more than one Fe–S cluster. The most extreme form is complex I (NADH-ubiquinone oxidoreductase) of the respiratory chain, which contains eight (eukaryotes) to nine (bacteria) Fe–S clusters (Hinchliffe and Sazanov, 2005). More complex structures with two or more of these simple Fe–S clusters substitute iron ions with heavy metals such as molybdenum, vanadium, nickel. Most well-known examples of such complex metalloclusters are the P-cluster of nitrogenase, H-cluster of bacteria, the FeMo cofactor, the nickel-containing cluster of bacteria, NiFe hydrogenases, xanthine dehydrogenase, plant aldehyde oxidase, plant nitrate reductase, and algal iron-only hydrogenases (Meyer, 2007).

There is no single common consensus motif binding for Fe–S clusters. However, some consensus motifs have been reported; especially, the conserved positioning of cysteine residues in the protein, for example, the CX4CX2CX ≈ 30C motif in plant and mammalian [2Fe–2S] ferredoxins and [4Fe–4S] clusters, is often coordinated by the consensus motif CX2CX2CX20–40C, which was originally defined in [4Fe–4S] ferredoxins, but seems to be present in many other members of the [4Fe–4S] cluster type. Frequently, a proline residue is located next to one of the cysteine residues. The lack of consensus motif makes it difficult to predict and discover the new Fe–S proteins based on their sequence information encoded in genomes. The various biophysical techniques available to study the structure and properties of Fe–S clusters are ENDOR, NMR, Mössbauer, EXAFS, and resonance Raman spectroscopies. These techniques provide valuable information mainly about the oxidation state, the type, and electronic and magnetic properties of the Fe–S clusters (Moulis et al., 1996). Many Fe–S proteins

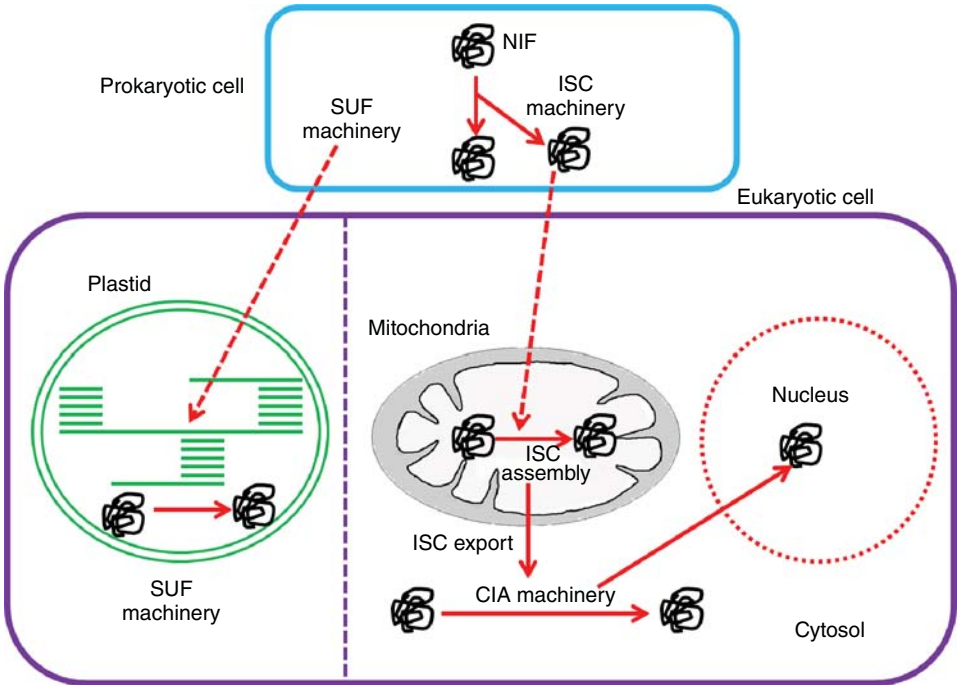


Figure 6.1 Three distinct types of machineries support the cellular Fe–S protein biogensis in prokaryotes and eukaryotes and their putative evolutionary origin. In prokaryotes: the NIF system present in nitrogen-fixing bacteria (*Azotobacter vinelandii*) is specialized in the assembly of the complex Fe–S protein nitrogenase, which is responsible for the conversion of N_2 to NH_3 in nitrogen-fixing bacteria (see Chapters 2 and 7). The ISC system is responsible for the generation of the majority of cellular Fe–S proteins particularly under normal and low oxygen concentrations. The SUF system is used preferentially under oxidative stress and iron limiting conditions. In eukaryotes, Fe–S protein exists in mitochondria, cytosol and the nucleus. The mitochondrial ISC assembly machinery is required for generation of all cellular Fe–S proteins. The ISC export apparatus and the CIA machinery are particularly required for the maturation of cytosolic and nuclear Fe–S proteins. The mitochondrial ISC assembly machinery is required for every generation of virtually all cellular Fe–S proteins. The mitochondrial ISC and SUF machineries were inherited by the eukaryotic cell from the bacterial endosymbiont that gave rise to modern mitochondria and plastids. These three different types of machineries are highly conserved from prokaryotes to eukaryotes.

are quite sensitive and are destroyed under oxidative conditions or exposure to air, NO, or H_2O_2. Most of the Fe–S proteins possess versatile electrochemical properties with reduction potentials ranging from +300 to –500 mV (Meyer, 2008).

6.4 FORMATION OF Fe–S CLUSTERS

The initial identification of proteins involved in Fe–S cluster assembly came from genetic and biochemical analysis of proteins specifically required for biological nitrogen fixation in *A. vinelandii* (Jacobson et al., 1989; see Chapter 2). Fe–S clusters can be assembled in apoproteins *in vitro* in the presence of sulfur, ferrous or ferric, and a thiol compound, for example, dithiothreitol. However, *in vivo* conditions are more complicated and require numerous key components. In addition, free sulfide and ferric iron are extremely toxic for cells; thus, they require the so-called Fe–S biogenesis machinery. Fe–S biogenesis is well documented in the model organisms *E. coli* and *S. cerevisiae*. The basic principles and key molecular players needed for Fe–S cluster formation are conserved almost in all kingdoms of life. In brief, a cysteine desulfurase produces sulfur from L-cysteine and iron and meets at the scaffold protein, which provides a molecular platform to form an Fe–S cluster, and finally this cluster is transferred to the target apoprotein.

6.5 DIVERSE FUNCTIONS OF Fe–S PROTEINS

Since the discovery of ferredoxins in the early 1960s, numerous proteins have been identified that contain Fe–S clusters known as Fe–S proteins. To date, more than 120 distinctive types of enzymes and proteins contain Fe–S clusters and have been proliferating greatly (Beinert et al., 1997). A summary of the types of biological Fe–S clusters, their functions, and localization are presented in Table 6.3. Since Fe–S clusters form the most ancient and functionally versatile prosthetic

Table 6.3 Known Fe–S proteins present in prokaryotes and eukaryotes (bacteria, yeast, plants, and animals), their cluster types, localization, and functions

Fe–S Proteins	Cluster Types	Localization	Functions
Aconitase	[4Fe–4S]	Mitochondria matrix	Citric acid cycle
Homoaconitase	[4Fe–4S]	Mitochondria matrix	Biosynthesis of lysine
Dihydroxy acid dehydratase	[4Fe–4S]	Mitochondria matrix	Biosynthesis of branched chain amino acids
Lipoate synthase	[2Fe–2S]? [4Fe–4S]	Mitochondria matrix	Biosynthesis of lipoic acid
Biotin synthase	[2Fe–2S]? [4Fe–4S]	Mitochondria matrix	Biosynthesis of biotin
Ferredoxin	[2Fe–2S]	Mitochondria matrix	Maturation of Fe–S proteins, biosynthesis of heme A, steroid biosynthesis in mammals (adrenodoxin)
Ferredoxin-like	[2Fe–2S]	Mitochondria matrix	Electron transport
Ferrochelatase	[2Fe–2S]	Mitochondria inner membrane	Heme biosynthesis (no cluster in yeast)
Complex I	8–9 cluster of [2Fe–2S], [3Fe–4S], [4Fe–4S]	Mitochondria inner membrane	Electron transport chain (NADH ubiquinone oxidoreductase)
Complex II	[2Fe–2S], [3Fe–4S], [4Fe–4S]	Mitochondria inner membrane	Electron transport chain (Succinate dehydrogenase)
Complex III	[2Fe–2S]	Mitochondria inner membrane	Electron transport chain (Ubiquinone cytochrome c oxidoreductase)
Cytokine-induced apoptosis inhibitor 1 (Dre2)	[2Fe–2S], [4Fe–4S]	Cytosol, Mitochondria inner membrane	?
DNA glycosylase	[4Fe–4S]	Nucleus	DNA glycosylase
Iron–sulfur cluster scaffold homolog (Isu1)	[2Fe–2S]	Mitochondria matrix, cytosol	Scaffold
Elp3	[4Fe–4S]	Nucleus	Histone acetyltransferase subunit of the elongation complex, cluster binds S-adenosyl-methionine
Isopropylmalate isomerase	[4Fe–4S]	Cytosol	Biosynthesis of leucine
Iron-regulatory protein 1	[4Fe–4S]	Cytosol	Post-transcriptional control of iron uptake, storage and use in mammals (Cytosolic aconitase')
Nucleotide-binding protein 1 (Nbp35)	[4Fe–4S]	Cytosol	Scaffold
Nucleotide-binding protein 2 (Cfd1)	[4Fe–4S]	Cytosol	Scaffold
Sulfite reductase	[4Fe–4S]	Cytosol	Biosynthesis of methionine, contains siroheme
Scaffold protein (Nfu1)	[4Fe–4S]	Mitochondria matrix, cytosol	Alternative scaffolding protein
Glutamate dehydrogenase	[4Fe–4S]	Cytosol	Biosynthesis of glutamate
Glutaredoxin 5		Mitochondria matrix	Cluster transfer
ABC protein Rli1 (Rli1)	[4Fe–4S] ?	Cytosol and nucleus	Biogenesis of ribosomes, rRNA processing, translation initiation
P-loop NTPase Nbp35	[4Fe–4S] ?	Cytosol and nucleus	Maturation of cytosolic and nuclear Fe–S proteins
Iron only Hydrogenase-like Nar1	[4Fe–4S]	Cytosol and nucleus	Maturation of cytosolic and nuclear Fe–S proteins
Iron-sulfur protein required for NADH-dehydrogenase (NUBPL)	[4Fe–4S]	Mitochondria matrix	Maturation of respiratory complex 1
MOCS1A	[3Fe–4S], [4Fe–4S]	Mitochondria matrix	Biosynthesis of Moco (molybdenum co-factor)
Dihydro-pyrimidine dehydrogenase	[4Fe–4S]	Cytosol	Degradation of pyrimidine nucleotides
CMP-N-acetyl-neuraminic acid	[2Fe–2S]	Cytosol	Biosynthesis of N-glycolyl neuraminic acid,
Xanthine dehydrogenase	[2Fe–2S]	Cytosol	Degradation of xanthine to urate, contains FAD and molybdopterin
Glutaredoxin 2	[2Fe–2S]	Mitochondria	Glutathione-dependent oxidoreductase

group, this allows them to participate in many vital processes of life (Fig. 6.2 a, b).

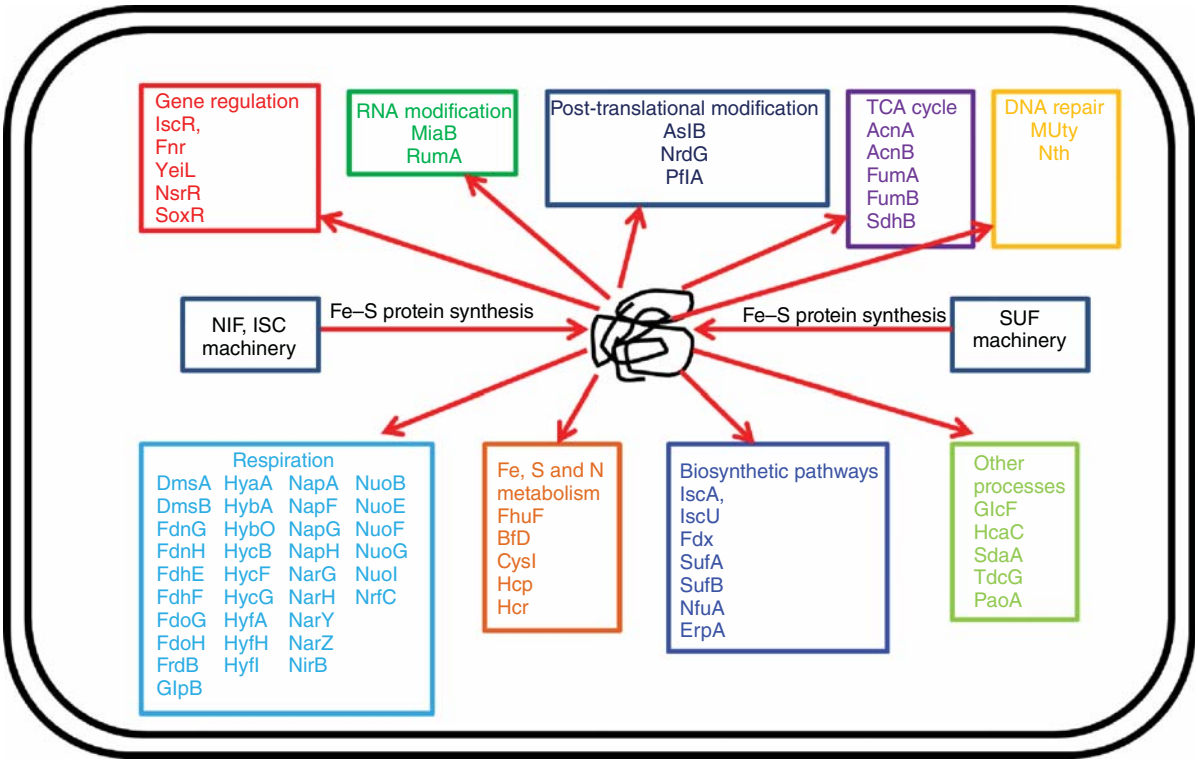
The main function of Fe–S proteins is electron transfer through the oxidation states of iron. Fe–S clusters have a wide range of redox potentials ranging from −500 to +300 mV (Meyer, 2008). Hence, Fe–S clusters can serve as excellent donors and acceptors of electrons in diverse biological processes: for instance, mitochondrial respiratory complexes I–III, *S*-adenosyl methionine enzymes, hydrogenases, ferredoxins, biotin synthase, photosystem I, and lipoate synthase. Therefore, the above example demonstrates that Fe–S proteins play an important role in electron transfer in the respiratory complexes of mitochondria and in the photosynthetic apparatus of chloroplasts (Booker et al., 2007). Fe–S clusters also play an essential role in sensing environmental or intracellular conditions to regulate gene expression such as the bacterial transcription factors FNR, IscR, and SoxR, which sense oxygen, Fe–S clusters, and superoxide or NO, respectively (Rouault, 2006; Wallander et al., 2006; Imlay, 2008; Volz, 2008). A well-studied example is cytosolic iron-regulatory protein 1 (IRP1), which regulates gene expression at the post-transcriptional level in mammals. The binding of IRP1 to 5′ end of IREs (iron responsive elements) causes inhibition of the ribosome scanning to the start AUG codon, resulting in a block in translation. However, the binding of IRP1 at 3′ end IREs protects mRNAs from nucleolytic degradation and leads to increased translation (Walden et al., 2006]. In addition, numerous catalytic functions have been known for bacterial and eukaryotic Fe–S enzymes involved in metabolism as well as for those playing a structural role (Meyer, 2008). Hence, Fe–S clusters also serve as the active sites of catalytic enzymes. For instance, the recently discovered Fe–S clusters in adenosine triphosphate (ATP)-dependent DNA helicases are involved in nucleotide excision repair (FANCJ, XPD, Rad3, and RTEL1) and telomere maintenance (Rudolf et al., 2006). Fe–S prosthetic groups are cofactors for sulfite and nitrite reductases, which are involved in sulfur and nitrogen assimilation, respectively, in the chloroplasts.

6.6 Fe–S CLUSTER BIOSYNTHESIS IN PROKARYOTES

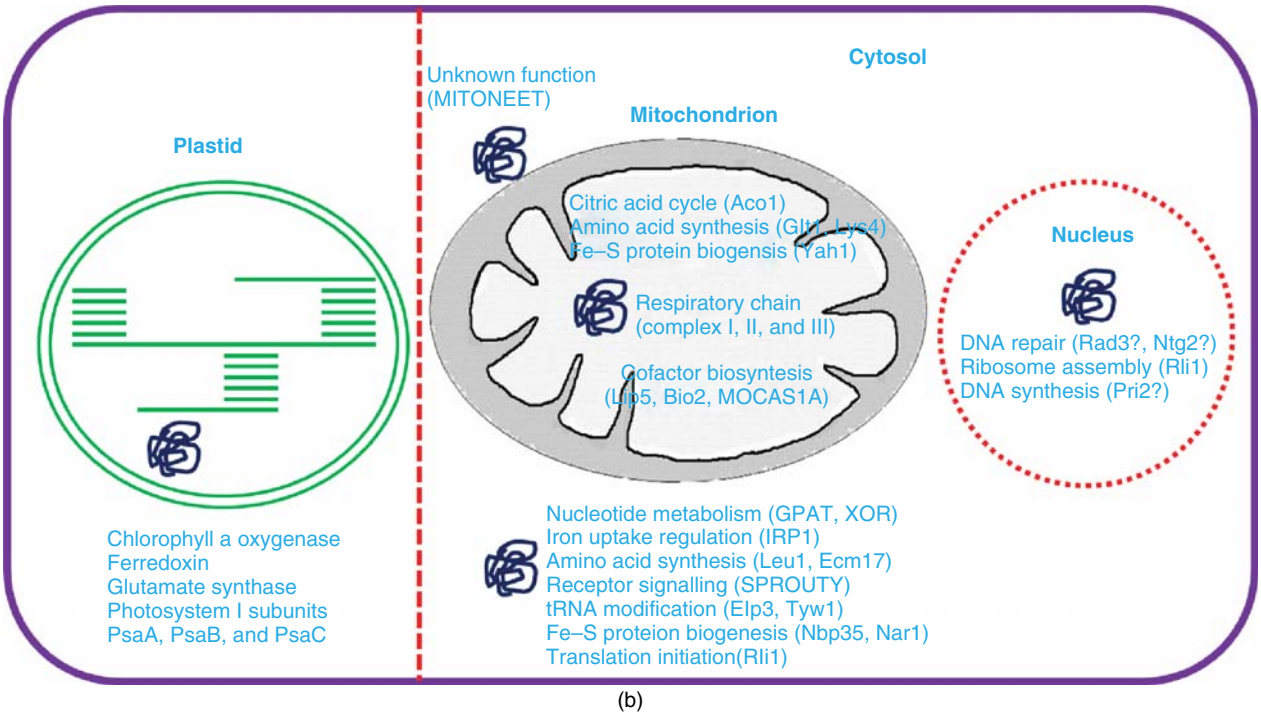
A search for nitrogenase maturation factors in *A. vinelandii* led to the discovery of the *nif* operon, *isc* operon, and subsequently *suf* operon. The biosynthesis of Fe–S clusters has been well documented in *E. coli* and *A. vinelandii*. However, the molecular mechanisms involved differ from organism to organism. Components of all of these systems are usually encoded by operons and exist almost in all microorganisms, supporting the idea that these systems are ubiquitous. Bacterial genome analyses revealed that the number and type of these operons varies from one microorganism to another. For instance, *E. coli* encodes the *isc* and *suf* operons, *A. vinelandii* contains the *nif* and *isc* operons, and *E. chrysanthemi* has the *nif*, *isc*, and *suf* operons. The components encoded for Fe–S cluster biogenesis by these operons also vary among microorganisms. One would expect that the increase in the availability of genome sequence data would provide more information about the Nif, Isc, and Suf systems. Indeed, bacterial genome analyses have identified new Fe–S cluster synthesis components. Interestingly, most of these genes have been implicated in the transfer of Fe–S clusters to the recipient target apoprotein. Here, we describe the general principle of Fe–S protein maturation in ISC and SUF systems. The assembly pathways of nitrogenases used by the NIF system are not addressed here (see Chapter 2).

6.7 ISC ASSEMBLY MACHINERY

In *E. coli*, the *isc* genes form an operon that encodes a regulator (IscR), a cysteine desulfurase (IscS), a scaffold (Iscu), an A-type protein (IscA), a Dnaj-like co-chaperone (HscA, HscB), and a ferredoxin (Fdx). General principles of Fe–S cluster biogenesis require that the Fe–S cluster assembles on a scaffold protein, which receives sulfur from a cysteine desulfurase and iron from an as-yet-unidentified source. Then, the preformed Fe–S cluster is transferred to a carrier protein, which delivers it to the final apotarget. Fe–S protein synthesis can be separated into two main steps. In the first reaction, the Fe–S cluster is initially assembled on the scaffold proteins IscU, which contain three conserved Fe–S cluster-coordinating cysteine residues (Kato et al., 2002). The assembly of the Fe–S cluster on IscU strictly depends on the function of IscS (cysteine desulfurase), which is a sulfur donor (Zheng et al., 1993). To date, the crystal structures of several desulfurases are known and illustrate a dimeric two-domain protein, one domain harboring the pyridoxal-phosphate-binding site and another a small domain containing the active-site cysteine that transiently carries the sulfur released from free cysteine as a persulphide. After the binding of iron to IscU, the Fe–S cluster forms but the molecular mechanism underlying this formation still remains to be clarified. The iron-binding protein frataxin (CyaY) is believed to function as a potential an iron donor by undergoing an iron-stimulated interaction with IsuU–IscS; however, its role is still uncertain and under debate (Gerber et al., 2003; Bencze et al., 2006; Layer et al., 2006; Wang and Craig, 2008). Fe–S cluster assembly on IsuU further depends on electron transfer from the [2Fe–2S] ferredoxin (Fdx), which receives its electrons from the mitochondrial ferredoxin reductase Arh1 and NADH. It is likely that the electron flow is needed for the reduction of the sulphan sulfur present in cysteine to the sulphide present in Fe–S clusters, but this remains to be established experimentally. An additional electron requirement was suggested for the fusion of



(a)



(b)

Figure 6.2 Functions and localization of Fe–S proteins in prokaryote (a) and eukaryotes (b). In eukaryotes, Fe–S proteins are located in mitochondria, cytosol, nucleus and plastids. They are involved in the basic cellular processes including gene regulation, DNA repair, Fe–S protein biogenesis, electron transfer, biosynthetic pathways and Fe, S and N metabolism. The functions of three Fe–S proteins located at the nucleus are not known (?). The names usually represent the abbreviations from the yeast *S. cerevisiae* and *E. coli.*

two [2Fe–2S] clusters to a [4Fe–4S] cluster by reductive coupling (Chandramouli et al., 2007; Unciuleac et al., 2007).

The second main step of biogenesis formally comprises the release of the Fe–S cluster from IscU, cluster transfer to recipient apoproteins, and its assembly into the apoprotein. However, these three partial reactions have not been separated experimentally so far. The overall process is specifically assisted by a dedicated chaperone HscA and HscB in an ATP-dependent manner. HscA recognizes and binds to a specific conserved motif of IscU (Leu-Pro-Pro-Val-Lys) and its interaction with the scaffold protein is regulated by the co-chaperone HscB. This complex formation and the involvement of HscB are thought to induce a structural change in IscU, thereby labilizing Fe–S cluster binding. Hence, it assists the cluster in dislocating and transferring to apoproteins (Cupp-Vickery et al., 2004; Dutkiewicz et al., 2004; Andrew et al., 2006; Chandramouli and Johnson, 2006; Bonomi, et al., 2008). As all available evidence suggests, assembly and release steps depend on the diverse conformational states of the scaffold, chaperone, and target apoproteins. Many of the Fe–S carrier proteins act as a step between cluster assembly on the scaffold and transfer to the target, but these carrier proteins remain to be defined. Fe–S cluster transfer from Isu1 to apoproteins is performed by the mitochondrial monothiol glutaredoxin Grx5; yet, its precise function remains to be elucidated. Fe–S cluster transfer to target recipient apoproteins varies, depending on the receiver apoprotein and the diverse environmental conditions that regulate the process by cellular demand. The above-mentioned ISC proteins are required for generation of all mitochondrial Fe–S proteins, but some biogenesis components play a more specific role. The interacting mitochondrial proteins Isa1, Isa2, and Iba57 (Table 6.1) are specifically involved in the maturation of a subset of Fe–S proteins that are members of the aconitase superfamily (Gelling et al., 2008; Muhlenhoff et al., 2011).

6.8 THE SUF MACHINERY IN BACTERIA AND PLASTIDS

It has been recently shown that *suf* systems are also involved in Fe–S cluster biosynthesis in bacteria, photosynthetic eukaryotes, and in the plastid of plants. Components of the SUF machinery are found in a variety of bacteria, including Archaea and photosynthetic bacteria. The *suf* genes are organized in an operon and consist of *sufA*, *sufB*, *sufC*, *sufD*, *sufS*, and *sufE* genes and particularly operate under specific conditions such as iron limitation and oxidative stress (Patzer and Hantke, 1999; Takahashi and Tokumoto, 2002; Ayala-Castro et al., 2008). SufA plays the role of a scaffold protein in the SUF system, which provides a platform to bring elemental sulfur and iron together into transient assembled clusters. SufB contains several conserved cysteine residues that can assemble an Fe–S cluster. Genetic, biochemical, and molecular biology studies in *E. coli* and *E. chrysanthemi* demonstrated that *sufC* plays an essential role in the formation of Fe–S clusters. Inactivation of SufC in *E. coli* and *E. chrysanthemi* results in a wide range of phenotypic changes that are related to iron homeostasis and oxidative stress. SufC is an ATPase located in the cytosol that is stimulated 100-fold by complex formation with SufB–SufD. Hence, SufC may assist the Fe–S cluster in detaching from SufB and subsequently transferring to apoproteins. In the SUF system, the cysteine desulfurase activity is served by the SufS/SufE complex, in which SufS acts similarly to bacterial IscS or NifS and mitochondrial Nfs1–Isd11. SufE stimulates SufS activity more than tenfold and allows the cysteine-bound persulphide intermediate on SufS to be transferred to a conserved cysteine residue on SufE from where it is passed on to scaffold proteins (Liu et al., 2005; Fontecave and Ollagnier-de-Choudens, 2008; Riboldi et al., 2009). The complex displays a drastically enhanced cysteine desulfurase activity as compared with SufS alone. Some of the *suf* genes also have been identified in archaebacterial genomes but, so far, nothing is known about Fe–S protein biosynthesis in Archaea.

6.9 Fe–S PROTEIN ASSEMBLY IN EUKARYOTES

In this section, we briefly summarize our current knowledge of the components of the various assembly systems and describe their respective mechanistic functions and functional interaction. Maturation of the nitrogenase complex in *A. vinelandii* led to the discovery of the *nif* operon. This discovery attracts the attention of several research groups working with Fe–S proteins and also influenced the first attempts to identify Fe–S cluster maturation in eukaryotes. The yeast *S. cerevisiae* has served as an excellent model organism with which to establish the preliminary details of the complex Fe–S biosynthesis machinery in eukaryotes. Genome analysis revealed that mitochondria contain a machinery that shares similarity with the bacterial ISC system. The striking similarities between the bacterial and mitochondrial ISC components and assembly mechanisms justify the conclusion that mitochondria inherited them from the bacterial endosymbiont that gave rise to modern mitochondria (Johnson et al., 2005; van der Giezen and Tovar, 2005). Over the last dozen years, several researchers have shown that not only the ISC assembly components but also the basic mechanisms of Fe–S cluster synthesis and transfer to target apoproteins are highly conserved from prokaryotes to eukaryotes.

6.10 MITOCHONDRIAL ISC ASSEMBLY MACHINERY

In eukaryotes, Fe–S proteins exist in mitochondria, the cytosol, plastids, and the nucleus. Mitochondria play a crucial role in the maturation of Fe–S clusters not only inside but also outside the organelle (Kispal et al., 1999; Lange et al., 2000). The mitochondrial ISC assembly machinery is required for the generation of all cellular Fe–S proteins. The biosynthesis of mitochondrial Fe–S proteins is accomplished by the ISC assembly machinery in three major steps. To date, more than 15 yeast proteins are known to assist this complex biosynthetic process. In the first step, the Fe–S cluster is initially synthesized de novo on a scaffold protein termed Isu1 and Isu2, which contains three cysteine residues that are crucial for de novo Fe–S cluster assembly. Isu1 is one of the best evolutionary conserved proteins and exists in almost all bacteria and virtually all eukaryotes (Yuvaniyama et al., 2000; Muhlenhoff et al., 2003; Raulfs et al., 2008). The first step critically depends on the function of the cysteine desulfurase complex Nfs1–Isd11 as a sulfur donor releasing sulfur from cysteine via persulfide intermediates (−SSH). The persulfide group is subsequently transferred from Nfs1 to Isu1, which is highly dependent on Isd11. The amino acid sequence of Isd11 is only moderately conserved but the protein is found basically in all organisms. Even though organisms lack the classical mitochondria (mitosomes and hydrosomes) they contain a homolog of Isd11, suggesting the fundamental role of Isd11 in Fe–S protein biogenesis (Adam et al., 2006; Wiedemann et al., 2006). The iron-binding protein frataxin (Yfh1) is believed to function as an iron donor by undergoing an iron-dependent interaction with Isu1 and may serve as a potential iron donor or an allosteric regulator of this step. Although the source of sulfur for Fe–S cluster formation has already been discovered, the source of iron is still under debate. However, mutations in the acidic ridge of Yfh1 impair both iron binding and the interaction with Isu1, indicating the functional significance of this region (Wang and Craig, 2008). Iron can be imported into the mitochondrial matrix only in its reduced form (Fe2+). This step uses the proton motive force as a driving source for membrane transport, which is assisted by the integral inner membrane proteins Mrs3 and Mrs4 (Zhang et al., 2005).

The second main step of biogenesis formally comprises the release of the Fe–S cluster from Isu1 and its transfer to recipient apoproteins by coordination with specific amino acid residues. The overall process is specifically assisted by a dedicated chaperone system comprising the Hsp70 ATPase Ssq1, the DnaJ-like co-chaperone Jac1, and the nucleotide exchange factor Mge1. Ssq1 binds to a small highly conserved motif (LPPVK) of Isu1. This complex formation stimulates the ATP-dependent hydrolysis of Ssq1 and dislocates the Fe–S cluster from Isu1. Jac1 is a co-chaperone and stimulates the ATPase function of Ssq1, thus assisting its transfer to apoproteins (Hoff et al., 2002; Dutkiewicz et al., 2004). The nucleotide exchange factor Mge1 serves to exchange the binding ADP for ATP to start a new cycle. The Fe–S cluster is released from Isu1 by the binding of an ATP-dependent Hsp70 chaperone Ssq1, its co-chaperone Jac1, and the nucleotide exchange factor Mge1 (Schilke et al., 2006). The Fe–S cluster may transiently be taken over by a monothiol glutaredoxin Grx5 toward apoproteins with the help of transient binding of the Fe–S cluster in a glutathione-containing complex (GSH) and finally its assembly into apoproteins by coordination with specific amino acid ligands. The above-mentioned proteins are required for the generation of all mitochondrial Fe–S proteins, for cytosolic and nuclear Fe–S protein biogenesis, and for transcriptional iron regulation. Thus, these proteins are called the "core ISC" assembly proteins. However, some proteins play a more essential and specific role. In the last step, some specialized proteins such as Isa1, Isa2, and Iba57 are specifically required for the maturation of the aconitase family members, for instance, aconitase-type (Aco1, Lys4) and SAM-dependent (Bio2, Lip5) Fe–S enzymes (Gelling et al., 2008; Muhlenhoff et al., 2011). The first two steps are required for maturation of all mitochondrial Fe–S proteins. Depletion of "core ISC" assembly proteins, for instance, Nfs1–Isd11, ferredoxin, or the chaperones leads to a severe impairment of Fe–S cluster insertion into cytosolic and nuclear target apoproteins such as Rli1, Leu1, Ntg2, or Rad3 (Kispal et al., 1999; Lange et al., 2001; Rudolf et al., 2006; Netz et al., 2012).

6.11 MITOCHONDRIAL ISC EXPORT SYSTEM

The mitochondria localized ISC assembly machinery is suggested to produce a still unknown component that is exported from the mitochondrial matrix to the cytosol, where it is required for efficient maturation of extramitochondrial Fe–S proteins. This suggests that the mitochondrial export system supports the synthesis of the Fe–S cluster in the cytosol and nucleus. The unknown component is predicted to be a sulfur-containing preassembled Fe–S cluster. The export reaction is accomplished by the ABC transporter Atm1 of the mitochondrial inner membrane. Another component required for the export reaction is the sulfhydryl oxidase Erv1, located in the intermembrane space. This enzyme has also been shown to catalyze the formation of disulphide bridges in the intermembrane space during Mia40-dependent protein import into the intermembrane space. The enzyme may deliver the electrons derived from the sulfhydryl oxidation process to either molecular oxygen or cytochrome *c*. Thus, Erv1 seems to perform multiple functions. A third component of the ISC export machinery is the tripeptide

glutathione. Apparently, depletion of GSH in yeast results in the downregulation of Atm1 or Erv1-led defective CIA Fe–S cluster synthesis, resulting in increased iron uptake in the cell and mitochondria. However, the maturation of mitochondrial Fe–S proteins is normal. Therefore, it seems that Atm1 plays a critical role in CIA Fe–S protein maturation and also in cellular iron regulation. Atm1, Erv1, and GSH have been described as the ISC export machinery (Kispal et al., 1999; Lange et al., 2001; Sipos et al., 2002).

6.12 CIA ASSEMBLY APPARATUS

The CIA machinery consists of seven known components. The CIA components do not share any sequence similarity with the ISC components, and their defect does not show any noticeable effects on the biosynthesis of mitochondrial Fe–S proteins. However, the basic mechanisms of Fe–S protein maturation and transfer to recipient apoproteins in model organisms have demonstrated that the entire process follows similar biosynthetic rules and is highly conserved in almost all eukaryotes. The first-known example of an essential CIA Fe–S protein is the ABC protein Rli1, which is involved in ribosome assembly and export from the nucleus (Kispal et al., 2005). Experimentally, CIA assembly can be split into two major steps. In the first step, the Fe–S cluster is assembled on the hetero-tetramer P-loop NTPases Cfd1 and Nbp35 serving as a scaffold. These two proteins are present in almost all eukaryotes and they contain three conserved cysteine residues. Two of these residues were shown to be essential for the function of Cfd1. Nbp35 possesses an additional Fe–S cluster at its N terminus with four essential cysteine residues that bind an ISC (Hausmann et al., 2005). This segment is essential for its function, yet its precise role remains elusive. An electron transfer chain consisting of NADPH, the diflavin reductase Tah18, and the Fe–S protein Dre2 is required for stable insertion of the Fe–S proteins into Nbp35 (Netz et al., 2010). However, the mechanism and precise molecular function of the electron transfer chain remain to be elucidated. The core mitochondrial ISC and export ISC machinery components are required for this step but the molecular details of this dependence still remain to be dissected in more detail. In a second step, the Fe–S cluster is transferred from the Cfd1–Nbp35 scaffold to target apoproteins, a reaction requiring the CIA proteins Nar1, Cia1, and possibly Cia2. Holo-Nar1 and the WD40 repeat protein Cia1 facilitate the dissociation of newly assembled Fe–S clusters from Cfd1-Nbp35 and their incorporation into cytosolic and nuclear apoproteins (Roy et al. 2003). Assembly of Fe–S proteins in the eukaryotic cytosol and nuclei requires the assistance of both the mitochondrial ISC assembly and a mitochondrial ISC export machinery. The molecular mechanisms of CIA protein functions are still poorly understood but are conserved in all eukaryotes. Recently, two other essential (nuclear) Fe–S proteins were identified, one (Rad3) with a function in nucleotide excision repair and the other (Pri2) with a function in RNA primer synthesis for DNA replication (Rudolf et al., 2006; Klinge et al., 2007). It seems that maturation of mitochondrial Fe–S proteins have been maintained during the evolution with an essential function in gene expression. This intimate connection between the endosymbiontic host and the bacterial ancestor of mitochondria may have been decisive in the maintenance of these organelles in eukaryotes. The crucial role of mitochondria in Fe–S protein synthesis has raised several questions about those organisms that lack classical mitochondria such as how and where these proteins are assembled in such organisms. These include diplomonads (*G. intestinalis*), microsporidia (*E. cuniculi*), Trichomonads (*Trichomonas vaginalis*), Apicomplexa (*C. parvum*), and *Entamoeba* (van der Giezen and Tovar, 2005; Embley and Martin, 2006). Cell-biological studies over the past years have identified small organelles in these amitochondriate species that are derived from the ancestral mitochondrion. It is now accepted that mitosomes descended from classical mitochondria by reductive evolution. Therefore, it is tempting to speculate that these organelles might accommodate ISC-like proteins. Genome analysis of amitochondriate species revealed the presence of homologs of the ISC, ISC export assembly, and CIA machineries. In support of these findings, the *G. intestinalis* proteins Nfs1 and Isu1 are located in mitosomes, which suggests that the other ISC proteins might be co-localized. Recently, hydrogenosomes of *T. vaginalis* have been shown to contain the ISC proteins Nfs1 and Isu1 (Tovar et al., 2003; Goldberg et al., 2008). These proteins are capable of assembling an Fe–S protein in vitro. These initial data suggest that both mitosomes and hydrosomes might be the place of Fe–S protein biogenesis.

6.13 CONCLUSION

Tremendous progress has been made in our understanding of the Fe–S protein cluster synthesis over the past years. The abundance of ISC, ISC export, SUF, and CIA assembly components has been identified and characterized in prokaryotes and eukaryotes. However, we still lack fundamental understanding of the function of many associated components at the biochemical and molecular levels. It would be intriguing to elucidate the mechanisms of Fe–S cluster transfer from the scaffold proteins to recipient apoproteins. The dissection must be into the partial steps and describe the precise function of the assisting component during each step. Solving of the three-dimensional structures of the distinct Fe–S cluster assembly components, specifically the holo forms of Fe–S scaffolds, will support the unraveling of the molecular mechanisms of *de novo* Fe–S cluster biogenesis.

In addition, the specificity and diverse needs of the various scaffold proteins by ISC and CIA biosynthetic machineries must be decoded to understand their precise individual roles. This will require combined studies and *in vitro* and *in vivo* investigations of the participating functional components, and therefore, will lead to a better understanding of the physiological significance of the suggested molecular mechanisms defined with purified components. Furthermore, it is important to reveal the conversion of [2Fe–2S] clusters to [4Fe–4S] clusters and illustrate the function of participating components. Another exciting area of future research will be the identification and biochemical and molecular characterization of the unknown component exported by Atm1 from mitochondria to cytosol. Moreover, the source of iron and sulfur in Fe–S protein assembly in the CIA and SUF system remains to be addressed. Additionally, we need to understand the link between Fe–S cluster maturation and iron homeostasis inside the cell. Finally, the fundamental process of life associated with Fe–S cluster synthesis would allow us to understand the physiological consequences of Fe–S protein defects and will lead to the development of therapeutic strategies in the treatment of Fe–S diseases. Recently, the global proteomics and genetic approaches in *E. coli* have identified the new candidate genes involved in Fe–S cluster metabolism, for example FdhD, which interacts with IscS and is involved in the maturation of Fe–S clusters containing formate dehydrogenase complex. In addition, the *ygfZ* and *folB* encoded for folate and mutation in *ygfZ* and *folB* have similar phenotypes of *isc* mutations. Detailed studies might lead to the finding of an unexpected link between folate and Fe–S cluster biosynthesis. Therefore, it is tempting to speculate on the greater diversity of the Fe–S biogenesis than expected, and new components of this complex machinery remain to be identified.

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Chapter 7

Biosynthesis of the Iron–Molybdenum Cofactor of Nitrogenase

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7.1 INTRODUCTION: DINITROGENASE AND APO-DINITROGENASE

Two major strategies for metal cofactor biosynthesis can be found in nature. In some cases the cofactor is assembled while directly attached to its target. The [4Fe–4S] cluster of the scaffold protein SufU and the nitrogenase [8Fe–7S] P-cluster are examples of *in situ* cofactor assembly (Malkin and Rabinowitz, 1966; Johnson et al., 2005; Rubio and Ludden, 2005; Fontecave and Ollagnier-de-Choudens, 2008; Albrecht et al., 2010; see Chapter 6). In the case of more complex metal cofactors, the *ex situ* approach is normally used. The iron–molybdenum cofactor (FeMo-co) of nitrogenase, the molybdenum cofactor (Mo-co) of nitrate reductase, and the H-cluster of [FeFe]-hydrogenase are examples of cofactors where *ex situ* assembly occurs (Rubio and Ludden, 2008; Shepard et al., 2011; Mendel, 2013; see Chapter 2). FeMo-co synthesis is completed outside the target enzyme in a biosynthetic pathway completely independent of the production of the structural polypeptides. Thus, FeMo-co needs to be inserted into the apo-enzyme in order to render the mature, active nitrogenase enzyme.

NifDK (also referred to as dinitrogenase or MoFe protein or nitrogenase component I) is a 230-kDa $\alpha_2\beta_2$ tetramer of the *nifD* and *nifK* gene products. The α and β subunits arrange as a pair of αβ dimers that are related by a twofold rotation axis. Despite low similarity between primary structures, both α and β subunits display a similar tertiary structure consisting of three domains each. NifDK contains two unique metal clusters per αβ-dimer: the P-cluster and FeMo-co (Chan et al., 1993; see Chapter 2).

The P-cluster is a [8Fe–7S] cluster in which two [4Fe–4S] cubanes share a sulfide atom in between. The P-clusters are located at the interface between the α and β subunits at around 12 Å below the protein surface (see Chapter 2). In the dithionite-reduced state, amino acid residues α-Cys^{88} and β-Cys^{95} provide the thiol groups bridging the two cubanes, whereas residues α-Cys^{62}, α-Cys^{154}, β-Cys^{70}, and β-Cys^{153} coordinate the remaining Fe sites in the P-cluster (unless otherwise noted, amino acid residue numbering corresponds to the *Azotobacter vinelandii* NifDK sequence). NifU and NifS are needed for the initial formation of two pairs of [4Fe–4S] clusters that serve as precursors to the P-clusters. In addition, the concerted action of both NifZ and NifH is required for the biosynthesis of the complete set of P-clusters. A NifDK protein with

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only one P-cluster, or with two [4Fe–4S] clusters (but no P-cluster), is obtained from deletion mutants lacking NifZ or NifH, respectively (Hu and Ribbe, 2011).

FeMo-co is a [Mo–7Fe–9S] cluster with a single carbide atom residing in the cavity formed by the six central Fe atoms (Schmid et al., 2002; Spatzal et al., 2011). One Fe and one Mo atoms cap both ends of the cofactor. The Mo atom is coordinated by the C-2 carbonyl and hydroxyl groups of the organic acid *R*-homocitrate (Fig. 7.1; see Chapter 2).

FeMo-co is buried within the α-subunit of each NifDK pair, 10 Å below the protein surface and 14 Å away from the P-cluster. Hydrophilic residues form the majority of the protein environment around FeMo-co, although a number of hydrophobic residues are required as well for cofactor positioning. Unlike the P-cluster, FeMo-co is ligated by only two NifD amino acid residues: α-His^{442} (which binds to the Mo atom) and α-Cys^{275} (which binds to the Fe atom located at the opposite end of the cluster) (Chan et al., 1993). Several other residues surrounding the cofactor binding site are selected to create a protein environment tailored for FeMo-co binding, such as α-Gly^{356} and α-Gly^{357} (which are needed to prevent steric hindrance with the metal cluster), α-Arg^{96} and α-Arg^{359} (which hydrogen bond to and stabilize the cofactor), or α-Gln^{191}, α-Glu^{440}, and α-Glu^{427} (which interact directly or through water molecules with the homocitrate moiety). As expected, residues α-His^{442}, α-Cys^{275}, and some other residues in the vicinity of FeMo-co are highly conserved across species.

Apo-NifDK (also denominated apo-dinitrogenase or apo-MoFe protein) refers to a cofactorless NifDK protein. Several forms of apo-NifDK have been reported to accumulate in the cell depending on the genetic background (Rubio and Ludden, 2005). For example, a Δ*nifH* mutation renders apo-NifDK lacking both the P-clusters and FeMo-co whereas a Δ*nifB* mutation renders apo-NifDK, which contains P-clusters but lacks FeMo-co. For the purpose of this chapter, we will use the term apo-NifDK when referring to the FeMo-co-less form found in Δ*nifB* strains (Schmid et al., 2002). This form of apo-NifDK can be readily activated by the simple addition of FeMo-co. In fact, apo-NifDK activation was used as an assay to isolate FeMo-co from pure NifDK protein (Pienkos et al., 1977).

Purified FeMo-co was found to be extremely sensitive to oxygen and unstable in protic solvents. Thus, FeMo-co extraction must be carried out in anaerobic N-methyl formamide (NMF) after denaturing and precipitating pure NifDK in a series of low and neutral pH solutions. FeMo-co isolated in this manner is stable indefinitely when stored as an anaerobic NMF solution under liquid nitrogen conditions. The isolation of FeMo-co is one of the seminal contributions to the field of nitrogenase biochemistry and to our understanding of complex metalloproteins assembly in general (see Chapter 2). FeMo-co isolation sets the basis for the *in vitro* FeMo-co insertion and the FeMo-co synthesis and insertion assays developed by Vinod Shah and now widely used in the field (Shah and Brill, 1977).

This chapter summarizes current knowledge on FeMo-co biosynthesis. As noted above, FeMo-co assembly occurs outside of NifDK in a complex biosynthetic pathway involving a series of biochemical activities that appear to

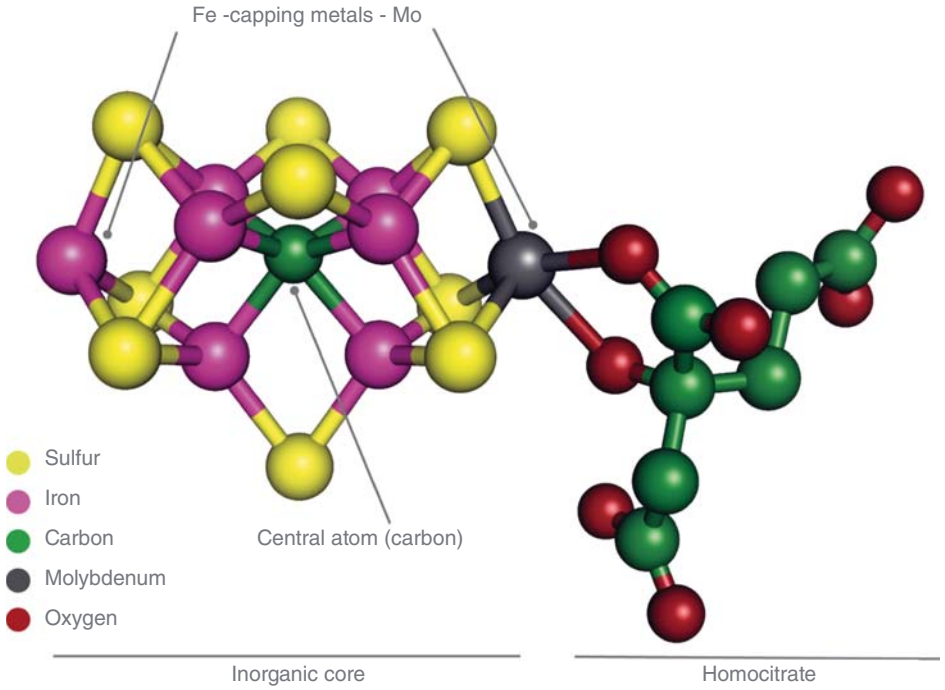

Figure 7.1 Structure of iron-molybdenum cofactor (FeMo-co) of nitrogenase.

be a common theme in complex metallocluster assembly in nature. FeMo-co biosynthesis requires enzymes, which provide substrates in the appropriate chemical forms and catalyze certain critical reactions such as carbide insertion, molecular scaffolds to aid in the step-wise assembly of FeMo-co, and metallocluster carrier proteins that escort FeMo-co biosynthetic intermediates in their transit between scaffolds (Table 7.1) (Rubio and Ludden, 2008; see Chapter 2).

Once fully assembled, FeMo-co is transferred from the FeMo-co "biosynthetic factory" into apo-NifDK, either via a hypothetical protein–protein interaction between NifEN and apo-NifDK (Hu and Ribbe, 2011) or mediated by FeMo-cofactor-binding proteins (Rubio and Ludden, 2008). The insertion of FeMo-co into apo-NifDK generates mature, functional holo-NifDK. Minor differences are observed in the βI, βII, βIII, αI, and αII domains of apo-NifDK upon FeMo-co insertion. However, the αIII domain undergoes major structural rearrangements. A comparison between the apo- and holo-NifDK structures revealed a widely conserved His triad (α-His^{274}, α-His^{442} and α-His^{451}) possibly involved in the formation of an insertion funnel in the structure of Δ*nifB* NifDK. The rearrangement of the αIII domain is hypothesized to generate an opening for FeMo-co insertion and to provide a positively charged path to drive FeMo-co entrance down to the cofactor binding site (Schmid et al., 2002). Site-directed mutagenesis studies on NifDK are consistent with the important role of the histidine residues along the insertion funnel to facilitate FeMo-co insertion (Hu and Ribbe, 2011).

7.2 NifU AND NifS

Many of the proteins involved in nitrogen fixation, including nitrogenase itself, are iron–sulfur (Fe–S) proteins. Given the large amount of Nif proteins expressed in nitrogen-fixing conditions, a specific [Fe–S] cluster biosynthetic system is found in model diazotrophic microorganisms in addition to the general [Fe–S] cluster biosynthetic machinery. This redundancy presents at least two major advantages: (i) it satisfies the high demand of [Fe–S] clusters needed for nitrogen fixation and (ii) it ensures that a deleterious mutation disturbing this specialized system only affects cell survival under diazotrophic growth conditions (Johnson et al., 2005)

NifU and NifS are required for the maturation of both NifH and NifDK. The critical observation to identify a role for these proteins was that whereas many mutations in *nif* genes affected either NifH or NifDK, mutations in either *nifU* or *nifS* resulted in a large decrease of activity in both nitrogenase components (Jacobson et al., 1989a). Since the presence of [Fe–S] clusters is common to both components it was proposed that NifU and NifS proteins had a role in the assembly of nitrogenase-specific [Fe–S] clusters. An additional key observation was the fact that although nitrogenase activity was severely affected in *nifU* and *nifS* mutants, it was not completely lost. This led to the identification of additional housekeeping NifU and NifS homologs, referred to as IscU and IscS, which were involved in supplying [Fe–S] clusters for general cellular functions (Zheng et al., 1998; see Chapter 6).

Table 7.1 Role of *nif* gene products in Mo-nitrogenase

Gene	Role of Gene Product
nifA	*nif*gene transcriptional activator
nifL	Anti-activator of *nif* gene transcription
nifY	In *K. pneumoniae* carries out same functions as *nafY*. Unknown function in *A. vinelandii*
nifU	Scaffold for [Fe–S] cluster biosynthesis. Forms complex with NifS
nifS	Cysteine desulfurase, serves as S donor for [Fe–S] cluster biosynthesis. Forms complex with NifU
nifX	Double role carrying NifB-co and serving as storage for VK-cluster (and probably other FeMo-co precursors)
fdxN	Donates electrons for FeMo-co biosynthesis
nifB	SAM-radical protein. Generates the complex FeS core of FeMo-co (including the central C atom)
nifV	Homocitrate synthase
nifQ	Donates molybdenum to the NifEN/NifH complex for FeMo-co biosynthesis
nifE	In combination with NifN, forms a scaffold protein involved in later steps of FeMo-co assembly
nifN	In combination with NifE, forms a scaffold protein involved in later steps of FeMo-co assembly
nafY	Double role stabilizing apo-NifDK and in FeMo-co insertion
nifZ	Involved in P-cluster formation
nifD	Dinitrogenase (MoFe protein) α-subunit. FeMo-co is buried within this subunit
nifK	Dinitrogenase (MoFe protein) β-subunit of. P-cluster is located at the interface of and subunits
nifM	Required for maturation of NifH (Fe protein). Similar to prolyl isomerases
nifH	Dinitrogenase reductase (Fe protein). Obligate electron donor to NifDK. Also required for P-cluster and FeMo-co syntheses

Genes from the nitrogen-fixing bacterium *A. vinelandii* are listed. The code is as follows: in bold are genes required for *in vivo* Mo-, V- and Fe-only nitrogenases; underlined are genes required for *in vitro* Mo-nitrogenase. Note: genes denoted in red together with those denoted in green are essential for full *in vivo* Mo-nitrogenase activity.

NifS is an 87-kDa pyridoxal phosphate (PLP)-containing homodimer (Zheng et al., 1993). Spectroscopic analyses showed that addition of L-cysteine altered the UV-visible properties of NifS, whereas none of the other biological amino acids was able to produce similar effects. Subsequent experiments demonstrated that NifS was a cysteine desulfurase that catalyzed a desulfurization reaction of L-cysteine, rendering L-alanine and sulfane as products. A highly conserved Cys^{325} residue located in the active site of the enzyme was found critical for NifS activity (Zheng et al., 1994). Based on sequence conservation, cysteine desulfurases are classified into two major groups. NifS falls within group I, with a SSGSAC(T/S)S conserved consensus sequence (Fontecave and Ollagnier-de-Choudens, 2008). Interestingly, a *cysE* homolog, encoding an *O*-acetyl serine synthase, the rate-limiting step for cysteine biosynthesis, is co-transcribed together with *nifS* (Evans et al., 1991).

NifU is a 66-kDa homodimer containing a stable [2Fe–2S] cluster per subunit (Fu et al., 1994). Primary sequence conservation analysis, site-directed mutagenesis experiments, and activity assays with separated purified domains confirmed the presence of three conserved domains in NifU (Agar et al., 2000). The central domain contains the permanent, redox-active, [2Fe–2S] cluster coordinated by four conserved cysteine residues, whereas the *N*-terminal and *C*-terminal domains present three and two conserved cysteine residues, respectively, for the assembly of transient [Fe–S] clusters (Smith et al., 2005). Spectroscopic and genetic analyses provided further evidence of formation of labile [2Fe–2S] clusters within both terminal domains of NifU, in reactions containing L-cysteine, Fe^{2+}, and NifS (Yuvaniyama et al., 2000).

A series of elegant experiments using apo-NifH as [Fe–S] cluster acceptor provided further details on the mechanism of NifU (Zheng et al., 1994; Dos Santos et al., 2004). NifS activity directs the assembly of transient [4Fe–4S] clusters on NifU, which are subsequently transferred to apo-NifH endowing protein activity. A NifUS complex formed during cluster assembly has been reported (Yuvaniyama et al., 2000), but NifS was not required for cluster transfer from the [Fe–S] donor NifU to the target apo-protein. Although *in vitro* loading of apo-NifH was possible simply by incubating with Fe^{2+} and S^{2-}, the reaction was significantly faster (at physiologically significant rates), specific, and more efficient (requiring only equimolar amounts of NifU) when using NifS, Fe^{2+}, and L-cysteine. All together, these results confirmed the role of NifS as donor of S^{2-} in order to sequentially load the scaffold NifU for the synthesis of simple [2Fe–2S] and [4Fe–4S] clusters required for maturation of both components of nitrogenase.

NifU and NifS are also involved in FeMo-co synthesis. Participation of these proteins as providers of [Fe–S] cluster substrates for FeMo-co biosynthesis was difficult to demonstrate because many FeMo-co biosynthetic proteins are Fe–S proteins themselves and mutations in *nifU* or *nifS* would have pleiotropic effects on the pathway. This puzzle was solved by investigating the capability of *nifUS* mutants to synthesize NifB-co, an early precursor to FeMo-co (see below). NifB-co biosynthesis was practically abolished in *nifUS* mutants (Zhao et al., 2007). Because NifU and NifS were shown not to be essential to render active NifB, the lack of NifB-co was attributed to a lack of [Fe–S] cluster precursors to assemble NifB-co (and hence FeMo-co).

NifB-co is a biosynthetic intermediate not only of FeMo-co but also of the FeV-co and FeFe-co of alternative nitrogenases. Consistently, *nifU* and *nifS* mutants were shown to be defective in Mo-nitrogenase, V-nitrogenase, and Fe-only nitrogenase activities (Kennedy and Dean, 1992).

7.3 NifB: FROM SIMPLE [Fe–S] CLUSTERS TO THE CORE OF FeMo-co

The *nifB* gene encodes a protein that includes an S-adenosyl methionine (SAM) radical motif CX_3CX_2C at the *N*-terminal region (Sofia et al., 2001). The *C*-terminal end of the protein comprises a NifX-like domain that is conserved in proteins with ability to bind FeMo-co and its biosynthetic precursors (Moreno-Vivian et al., 1989; Rubio et al., 2002). As mentioned above, NifB participates in an early biosynthetic step that is common to FeMo-co, FeV-co, and FeFe-co biosyntheses. Therefore, *A. vinelandii* mutants lacking *nifB* were incapable of diazotrophic growth under all conditions tested (Joerger and Bishop, 1988; Rodriguez-Quinones et al., 1993). Consistently, regulation of *nifB* expression by transcriptional activators of all three nitrogenases, NifA, VnfA, and AnfA, has been reported (Drummond et al., 1996).

NifB catalyzes the conversion of simple [2Fe–2S] or [4Fe–4S] clusters, donated by NifU, into a complex [Fe–S] cluster in a reaction that involves radical chemistry (Curatti et al., 2006). Interestingly, the metabolic product of NifB, termed NifB-co, was purified and studied before purification of active NifB was accomplished (Shah et al., 1994). NifB-co comprises the central [6Fe–9S–C] core of FeMo-co but does not contain a heterometal (e.g., Mo) or homocitrate (Shah et al., 1994; George et al., 2008). Early experiments showed that NifB-co served as precursor to FeMo-co in the *in vitro* FeMo-co synthesis assay (Shah et al., 1994) and that it was the source of most (if not all) Fe and S present in FeMo-co (Allen et al., 1995).

The NifB protein was first purified from *A. vinelandii* cells (Curatti et al., 2006). Isolated NifB was a 110-kDa homodimer containing ca. 12 Fe atoms and exhibiting a

UV–visible spectrum that is typical of [Fe–S] proteins. Changes in the NifB redox state and incubation with SAM altered the properties of its UV–visible spectrum, as expected for a redox-responsive SAM radical protein. Isolated NifB did not carry NifB-co nor was it readily active in supporting *in vitro* FeMo-co synthesis. However, after incubation with Fe^{2+} and S^{2-}, the Fe content increased to ca. 18 atoms and NifB became active (Curatti et al., 2006), providing the first demonstration of complete *in vitro* FeMo-co synthesis from its atomic components. The work of Curatti also laid the ground work for further mechanistic experiments by showing that radical chemistry was absolutely required for NifB activity. Later on, it was shown that the carbide atom at the center of FeMo-co had its origin in the methyl group of SAM (Wiig et al., 2012).

An interesting observation is that NifB-co could be inserted in the place of FeMo-co into nitrogenase *in vitro*. This artificial NifDK/NifB-co complex was capable of proton and acetylene reduction but not nitrogen fixation (Soboh et al., 2010). This observation emphasizes the importance of carrier proteins in redirecting metal precursors to the appropriate target proteins *in vivo* (see below).

7.4 NifQ: DIRECTING Mo TO FeMo-co SYNTHESIS

NifDK represents up to 5% of the total cellular protein content accumulated under diazotrophic growth. Thus, nitrogen-fixing *A. vinelandii* cells must cope with a large demand for Mo, a low abundance transition metal (1–2 ppm in soils). *A. vinelandii* produces siderophores, low molecular weight molecules with high affinity for metals, to aid in Mo (and Fe) acquisition (Kraepiel et al., 2009). Unfortunately, siderophores can bind to other metals, such as W, which can eventually be incorporated into FeMo-co rendering inactive nitrogenase (Siemann et al., 2003). To discriminate against tungstate, *A. vinelandii* carries ABC transport systems that are highly specific for molybdate (Mouncey et al., 1995). Three copies of the *modABC* operon are found in the *A. vinelandii* genome (Setubal et al., 2009), as opposed to a single copy in the closely related bacterium *Pseudomonas stutzeri*.

A. vinelandii has a unique Mo-accumulation system based on a Mo storage (MoSto) protein. MoSto is a $\alpha_3\beta_3$ heterohexamer of the *mosA* and *mosB* gene products with capacity to store up to 100 Mo atoms (Fenske et al., 2005) in the form of complexes of polynuclear oxoanions (Schemberg et al., 2007). In addition, cellular systems are in place to keep Mo homeostasis and to direct it to the corresponding Mo-dependent enzymes (Fig. 7.2). The molbindin ModG appears to be responsible for directing Mo to nitrogen assimilation pathways, such as nitrate reductase or nitrogenase (Mouncey et al., 1995). NifO has been related to Mo balance between nitrate reductase and nitrogenase. It was suggested that NifO would direct Mo toward FeMo-co synthesis, thus impairing development of nitrate reductase activity (Gutierrez et al., 1997).

The *nifQ* gene was identified by screening Nif mutants, the phenotype of which could be reverted by a large excess of molybdate or cysteine in the medium (Imperial et al., 1985). Although *nifQ* mutants did not accumulate molybdate they were not impaired in molybdate transport or in the activity of alternative nitrogenases (Joerger and Bishop, 1988; Rodriguez-Quinones et al., 1993) or Mo-co-containing enzymes, such as nitrate reductase (Pienkos et al., 1977).

NifQ proteins are found in all diazotrophic species of the Proteobacteria phylum, with the exception of some Rhizobia. NifQ proteins do not contain molbindin domains or show sequence similarity to MosA or MosB. They do contain a highly conserved *C*-terminal putative metal-binding motif $CX_4CX_2CX_5C$.

As isolated from *A. vinelandii,* NifQ was a monomeric 20-kDa, oxygen-sensitive protein, containing ca. 3 Fe atoms and 0.30 Mo atoms per monomer. NifQ displayed a UV–visible spectrum typical of (Fe–S) proteins. Electronic paramagnetic resonance (EPR) and electron spin echo (ESE)-EPR analyses revealed that NifQ carried a novel redox-responsive [Mo-3Fe–4S] cluster (Hernandez et al., 2008). *In vitro* FeMo-co synthesis assays with purified components demonstrated that NifQ served as a unique Mo source for FeMo-co synthesis. Comparison of Mo-content in Nif proteins before and after the FeMo-co synthesis reaction revealed that, only in the presence of NifH, Mo was effectively mobilized from NifQ to NifEN, demonstrating that all three proteins were required for Mo transfer (Hernandez et al., 2008).

The exact reaction(s) carried out by NifQ are not known. The complete processing of Mo from molybdate (Mo^{VI}) to the state found in FeMo-co (Mo^{IV}) requires at least three chemical transformations: (i) replacement of O ligands by S ligands, (ii) reduction of Mo from Mo^{VI} to Mo^{IV}, and (iii) insertion into an [Fe–S] environment. It has been suggested that the role of NifQ could be related to some (or all) of these changes (Hernandez et al., 2009).

7.5 NifV AND THE INCORPORATION OF HOMOCITRATE INTO FeMo-co

The *nifV* gene product is a homocitrate synthase that catalyzes the condensation of acetyl coenzyme A and α-ketoglutarate to render *R*-homocitrate (Zheng et al., 1997). The *nifV* mutants exhibited slow diazotrophic growth rates (McLean et al., 1983), a phenotype that could be reverted *in vivo* by supplementing the growth medium with homocitrate (Hoover et al., 1988). V- and

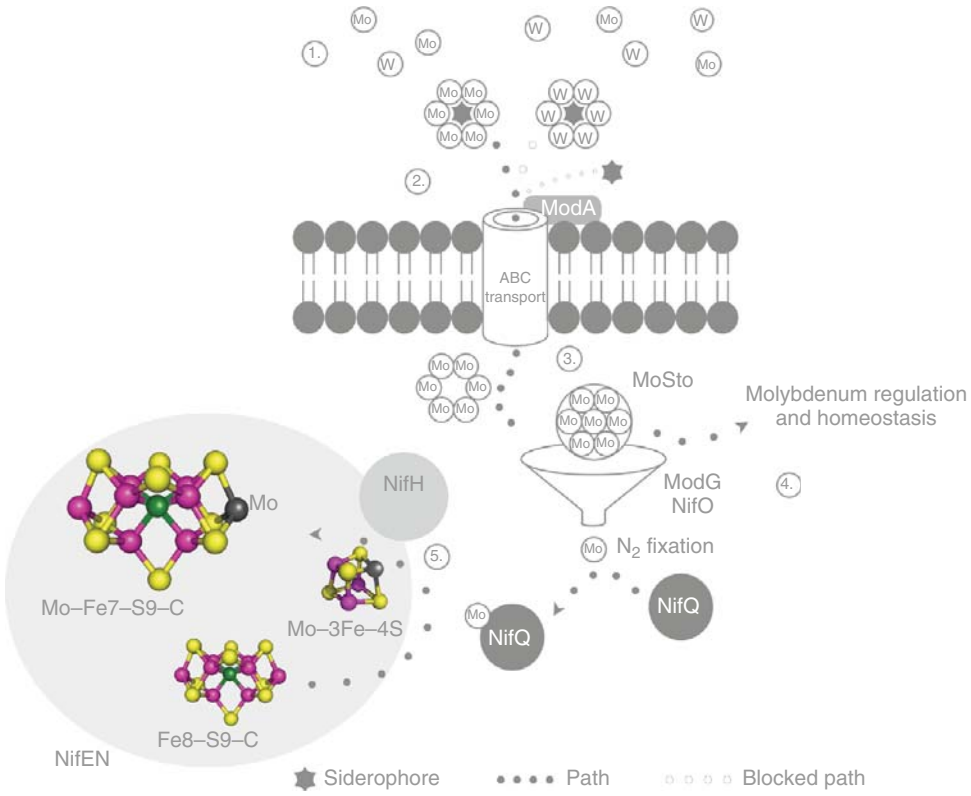

Figure 7.2 Molybdenum trafficking to FeMo-co can be divided into in five processes: (1) molybdate harvesting, (2) molybdate transport and discrimination against tungstate, (3) molybdenum accumulation and homeostasis, (4) molybdenum sorting to the appropriate pathway, and (5) molybdenum insertion into the cofactor.

Fe-nitrogenase-dependent growth was also impaired in these mutants (Kennedy and Dean, 1992), indicating that homocitrate was part of the FeV-co and FeFe-co cofactors as well.

Klebsiella pneumoniae nifV mutants have been shown to incorporate citrate into a non-functional form of FeMo-co *in vivo* (Liang et al., 1990). The situation was more complex in *A. vinelandii* where a mixture of organic acids replacing homocitrate in the cofactor was found (Ludden et al., 2004). *In vitro* FeMo-co synthesis assays carried out with analogous organic acids in the place of homocitrate resulted in the synthesis of cofactors with altered catalytic properties (Hoover et al., 1989). It is not clear how the nitrogen-fixing cell manages to discriminate between homocitrate and other analogous organic acids during FeMo-co synthesis. Homocitrate incorporation occurs within NifEN, presumably after Mo incorporation has taken place (Rangaraj and Ludden, 2002). It is possible that discrimination occurs within the NifEN/NifH complex. It is also possible that homocitrate concentration in the cell was so high that it would preclude incorporation of other organic acids.

7.6 NifEN: A NODE IN THE FeMo-co BIOSYNTHETIC PATHWAY

NifEN is a 200-kDa $\alpha_2\beta_2$ heterotetramer of the *nifE* and *nifN* gene products that carries two identical [4Fe–4S] clusters at the interface of both subunits (Goodwin et al., 1998). If isolated from the appropriate genetic backgrounds, NifEN preparations exhibit trapped FeMo-co biosynthetic intermediates (see below) (Hu et al., 2005; Soboh et al., 2006). NifEN is absolutely required for FeMo-co synthesis *in vivo* (Jacobson et al., 1989b) and *in vitro* (Curatti et al., 2007).

NifEN displays high similarity with NifDK at several levels, including amino acid sequence similarity (Brigle et al., 1987), position of their metal clusters within the protein (Kaiser et al., 2011), and the ability to catalyze acetylene and azide reduction albeit at very low rates (Hu et al., 2009). It was the amino acid sequence similarity of NifEN to NifDK that led to the proposal of NifEN acting as molecular scaffold for FeMo-co biosynthesis (Brigle et al., 1987).

NifEN acts as central node of the FeMo-co biosynthetic pathway, where additional Fe, Mo, and homocitrate are incorporated into NifB-co (Fig. 7.3) (Rubio and Ludden,

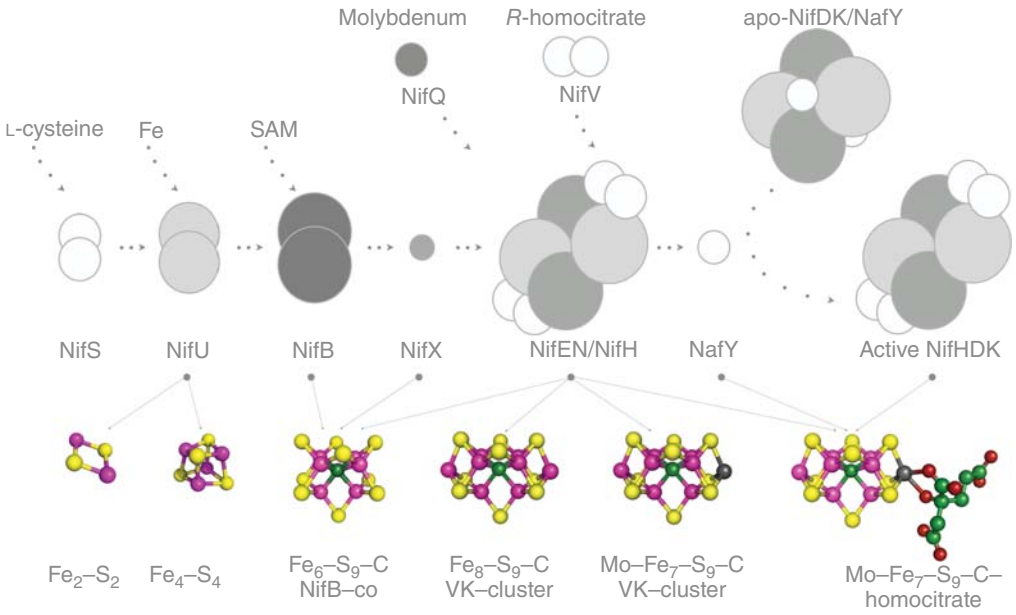

Figure 7.3 FeMo-co biosynthesis. This schematic model illustrates the enzymatic machinery (above) involved in different steps of FeMo-co assembly (below). Early steps involve NifS, NifU, and NifB for the assembly of NifB-co, the central Fe–S core of FeMo-co, and its transfer to the NifEN scaffold via NifX. Maturation to FeMo-co occurs within a putative NifEN/NifH complex by sequential addition of Fe, Mo, and homocitrate.

2008). Briefly, NifB-co is transferred from NifX to NifEN, where it is converted into the VK-cluster (named after Dr. Vinod K. Shah) (Hernandez et al., 2007). Although both NifB-co and the VK-cluster lack Mo and homocitrate and serve as precursors to FeMo-co, there are some differential properties that indicate they are not the same precursor. First, while NifB-co is EPR silent, the VK-cluster shows EPR signals both in reduced and oxidized states (Hernandez et al., 2007). Second, EXAFS analysis suggests that NifB-co is no larger than the central [6Fe–9S–C] core of FeMo-co (George et al., 2008), whereas the VK-cluster was proposed to be a larger [8Fe–9S] cluster (Hu et al., 2006). The recently solved NifEN crystal structure confirmed the assignment of 8 Fe atoms for the VK-cluster. Third, NifEN-mediated Fe incorporation into NifB-co at capping positions external to the [6Fe–9S–C] core was achieved *in vitro* (Rubio et al., unpublished results).

In addition to the VK-cluster, NifEN purified from a Δ*nifH* background has been shown to contain Mo in a separate [Mo–3Fe–4S] cluster environment (George et al., 2007). Occupancy levels for this cluster were low and dependent on the purification method used, probably due to cluster instability (Soboh et al., 2006). Nevertheless, it was shown to serve as Mo source during FeMo-co synthesis *in vitro*. The composition of this cluster resembles the one found in NifQ preparations and, since NifQ has been shown to be able to transfer Mo to NifEN *in vitro* (Hernandez et al., 2008), a logical proposal is that the [Mo–3Fe–4S] cluster within NifEN derives from the NifQ cluster. Another possibility is that this cluster represents a NifQ-independent Mo insertion pathway that would operate with lower efficiency. This pathway would be responsible for the reversion of the *nifQ* mutant phenotype by the presence of 1000-fold molybdate into the growth medium (Imperial et al., 1984; 1985).

Interestingly, NifEN is able to substitute for the homologous VnfEN protein of the V-nitrogenase (Wolfinger and Bishop, 1991). This finding raises questions regarding the specificity of NifEN in Mo insertion into FeMo-co and opens the possibility of other elements providing this specificity (Hernandez et al., 2009).

Finally, NifEN appears to be the site where homocitrate is incorporated into the cofactor in a reaction that requires NifH.

7.7 NifH: NITROGENASE MOONLIGHTING PROTEIN

NifH (also referred to as dinitrogenase reductase, Fe protein or nitrogenase component II) is the obligate electron donor to NifDK. NifH is a 60-kDa homodimer of the *nifH* gene product. The NifH structure revealed a twofold symmetric enzyme with $Mg^{2+} \cdot ATP$ binding sites located at the dimer interface within each monomer. Two cysteine residues from each NifH subunit coordinate a single [4Fe–4S] cluster at the subunit interface (Georgiadis et al., 1992). NifH undergoes conformational changes during $Mg^{2+} \cdot ATP$ binding and hydrolysis in a process coupled to electron transfer from the [4Fe–4S] cluster of NifH to the P-cluster of the NifDK component (Lanzilotta et al., 1998).

Three accessory proteins are necessary to synthesize active NifH, namely, NifU, NifS, and NifM (Jacobson et al., 1989a). NifM is similar to prolyl isomerases and has been proposed to induce a conformational change on NifH that precedes incorporation of its [4Fe–4S] cluster (Gavini et al., 2006). NifU and NifS are involved in the assembly and delivery of the NifH [4Fe–4S] cluster (Dos Santos et al., 2004).

NifH is a moonlighting protein with at least three essential roles in the nitrogenase system: (i) it is required for electron transfer to the NifDK component during catalysis, (ii) it is required to assemble P-clusters from pairs of [4Fe–4S] cluster precursors, and (iii) it is essential to FeMo-co synthesis, in which process it probably plays multiple roles.

Not all of NifH capabilities are required for the performance of all its functions. Many lines of evidence show that $Mg^{2+} \cdot ATP$ hydrolysis and electron transfer are required for catalysis but not for P-cluster or FeMo-co biosynthesis. First, *nifM* mutants were shown unable to fix nitrogen but able to support FeMo-co biosynthesis (Roberts et al., 1978). Second, [4Fe–4S] cluster-deficient apo-NifH (generated by chemical treatment of NifH to remove the metal clusters) was able to participate both in P-cluster synthesis and in FeMo-co synthesis (Rangaraj et al., 1997). Third, NifH variants with altered properties of $Mg^{2+} \cdot ATP$ binding and/or hydrolysis could carry out FeMo-co synthesis (Gavini and Burgess, 1992; Rangaraj et al., 1999). On the other hand, more recent experiments indicate that NifH must be able to hydrolyze $Mg^{2+} \cdot ATP$ and to transfer electrons in order to be active in FeMo-co biosynthesis (Hu et al., 2006).

NifH is absolutely required for FeMo-co biosynthesis. Neither Mo nor homocitrate is incorporated into the cofactor in the absence of NifH. However, the exact mechanism by which NifH exerts its role remains unclear. NifEN and NifH are able to interact transiently with each other (Rangaraj et al., 1999) and, in fact, Mo transfer from NifQ to NifEN occurs only in the presence of NifH (Hernandez et al., 2008). It has been proposed that NifH would play its role of facilitating Mo insertion into the VK-cluster simply by docking with NifEN and exerting some sort of conformational change on it (Rubio and Ludden, 2008).

The proposal of NifH being the element that selectively incorporates Mo into FeMo-co discriminating against other heterometals has long been discussed. Several observations do not support a role for NifH and other dinitrogenase reductases in specifying the heterometal to be inserted into the cofactor and point to other proteins (e.g., NifQ) being potentially responsible for heterometal discrimination. VnfH, the equivalent protein in V-nitrogenase, could replace NifH in FeMo-co biosynthesis (Chatterjee et al., 1997). Similarly, AnfH of the Fe-only nitrogenase supported FeMo-co synthesis *in vivo* (Gollan et al., 1993). Bishop and collaborators proved that NifH was able to support V-dependent diazotrophic growth in the absence of VnfH (Joerger et al., 1990).

Finally, it is well known that NifH must be present to render homocitrate-containing FeMo-co (Rangaraj and Ludden, 2002; Hu et al., 2006). However, incorporation of homocitrate into an isolated Mo-containing FeMo-co precursor has not yet been reported. Thus, a direct role for NifH in homocitrate incorporation into FeMo-co precursor remains hypothetical.

7.8 METALLOCLUSTER CARRIER (ESCORT) PROTEINS

Once a cofactor has been synthesized on a scaffold, it needs to be transferred to its target protein. When prosthetic groups are very labile and oxygen sensitive, direct diffusion is unlikely and the metal clusters are expected to be always protein-bound within the cell (Rubio et al., 2002). Additionally, there is a rapid demand for nitrogenase synthesis during diazotrophic growth, which, taken together, might explain the existence of proteins involved in metallocluster delivery.

The *nifX* gene is clustered into a single operon together with *nifEN*. The *nifENX* gene cluster is in fact widespread among bacteria, suggesting that three gene products have a related role. NifX is a ca 17-kDa single-domain protein. Although unable to bind ^{55}Fe or ^{99}Mo (Rangaraj et al., 2001; Rangaraj and Ludden, 2002) or assemble an [Fe–S] cluster, the product of the *nifX* gene has been shown to be able to ligate FeMo-co and FeMo-co precursors (Hernandez et al., 2007). Early studies speculated on a role of NifX in the incorporation of homocitrate into a FeMo-co precursor (Rangaraj and Ludden, 2002), or as a negative regulator of *nif*-gene expression in response to $[NH_4^+]$ and O_2 (Gosink et al., 1990). However, recent *in vitro* experiments have demonstrated different roles for NifX (Hernandez et al., 2007). First, it would work as donor of at least two FeMo-co precursors (NifB-co and VK-cluster) to NifEN. NifX and NifEN do not form a stable protein complex, but a transient interaction occurs for the metal cluster exchange to happen. Second, NifX would function as storage of FeMo-co precursors, redirecting labile metal clusters to NifEN. This might be especially relevant to buffer the flux of FeMo-co precursors under stress conditions, thus minimizing metal cluster losses.

NifX-like domains are present in a group of nitrogenase-related proteins, and thus serve to define a family of nitrogenase cofactor binding proteins, including VnfX and the *C*-terminal domains of NifB, NifY, NafY, and VnfY. NafY is probably the best characterized among them (Homer et al., 1995; Rubio et al., 2002; Rubio et al., 2004).

NafY is a ca. 26-kDa two-domain protein with a double role in apo-NifDK stabilization and in FeMo-co

insertion into apo-NifDK. Two functional domains can be defined in NafY, with each role mostly assigned to each domain. First, the 12-kDa *N*-terminal domain is sufficient to bind apo-NifDK in the absence of the rest of the protein. NMR solution structure of the *N*-terminal domain of NafY revealed that it contained a sterile alpha motif domain, a structure frequently involved in protein–protein interactions (Hernandez et al., 2011). This domain represented the first apo-NifDK binding structure known, other than NifH, and exhibited a novel fold for apo-NifDK binding, different from what is observed in the NifH structure (Georgiadis et al., 1992). Interestingly, excess of *N*-terminal NafY domain or full-length NafY had a negative effect on apo-NifDK reconstitution *in vitro*. Second, the 14-kDa *C*-terminal domain was shown to bind FeMo-co autonomously. The crystal structure of the core domain of NafY (defined as the *C*-terminal domain missing the last 13 amino acid residues) represents the only known FeMo-co binding fold different from that of NifDK (Dyer et al., 2003). Mutational analyses indicated direct implication of the His^{121} residue in FeMo-co binding (Rubio et al., 2004). These results suggest a model with a series of histidine residues involved in FeMo-co insertion into apo-NifDK. FeMo-co-bound to NafY via His^{121} would be donated to α-His^{362} (at the entrance of the insertion funnel in apo-NifDK), followed by entry into the positively charged environment created by the His triad (α-His^{274}, α-His^{442} and α-His^{451}) and finally donation to α-His^{442}, as one of the ligating residues of FeMo-co in NifDK (Schmid et al., 2002).

Given the low affinity of NafY for NifB-co and the ability of apo-NifDK to bind NifB-co (Soboh et al., 2010), this might be a physiological mechanism to couple FeMo-co synthesis to apo-NifDK activation, while preventing insertion of biosynthetic intermediates into the nitrogenase active site.

NifX and NafY are not strictly essential for *in vitro* FeMo-co synthesis or *in vivo* diazotrophic growth under standard laboratory conditions (Rubio et al., 2002; Curatti et al., 2007). However, several caveats need to be considered in order to appreciate their relevance. First, functional overlap among members of this family complicates the finding of a phenotype in deletion mutants. A BLAST search for NifX-like sequences reveals two additional homologs in the genome of *A. vinelandii* (Setubal et al., 2009), in addition to the above-mentioned members of this family. Thus, functional redundancy might obscure the observation of a phenotype in single mutant strains. Second, diazotrophic growth experiments of deletion mutant strains are typically carried out under optimal laboratory conditions. This might preclude the observation of a phenotype present under nutrient-limited environmental growth conditions. For instance, the double Δ*nifX* Δ*nafY* mutant or the triple Δ*nafY* Δ*nifY nifX::kan* mutant were impaired in diazotrophic growth under Mo starvation conditions (Rubio et al., 2002). Similar observations indicating the requirement of NifX under Fe-depleted conditions were reported in *Herbaspirillum seropedicae* (Klassen et al., 2003). Third, *in vitro* experiments proved the additive stimulatory effect of NifX and NafY on FeMo-co biosynthesis when present in the reaction mixture (Curatti et al., 2007). NifX and NafY were not required for NifB-co synthesis, but were able to independently increase apo-NifDK activation. Fourth, proteins involved in metal cluster storage and delivery have been described in other cofactor biosynthetic pathways, including the Mo-co carrier protein in *Chlamydomonas reinhardtii* (Fischer et al., 2006), the IscA and ErpA carriers in *E. coli* (Pinske and Sawers, 2012), and the mammalian MMS19 protein for [Fe–S] cluster assembly (Gari et al., 2012), to name a few. Similarly, enzyme-specific chaperones relevant to metal cofactor insertion into multisubunit metalloenzymes have been reported in other systems, such as the NarJ chaperone from *E. coli* (Vergnes et al., 2006) and the copper superoxide dismutase from *Saccharomyces cerevisiae* (Culotta et al., 1997). Hence, it is not surprising to find proteins with similar roles in the FeMo-co biosynthetic pathway.

7.9 CONCLUSION

The Mo-nitrogenase carries at its active site an FeMo-cofactor that is essential for nitrogen fixation activity. FeMo-co biosynthesis is a complex process involving a number of *nif* gene products that function as molecular scaffolds, metallocluster carriers, or substrate providers. Findings from biochemical and genetic studies using model organisms should be taken into account when designing a strategy to transfer *nif* genes into crop plants (e.g. cereals; see Chapter 108). The products of at least six genes: NifB, NifE, NifN, NifH, NifD and NifK have been shown to be absolutely required for FeMo-co biosynthesis and nitrogenase activity both *in vivo* and *in vitro*. On the other hand, it is likely that the products of some genes that are required for FeMo-co biosynthesis *in vivo* in model organisms could be replaced by the activities of plant counterparts. This group would include NifU, NifS, FdxN, NifQ and NifV, which provide simple [Fe–S] clusters, electrons for FeMo-co synthesis, molybdenum in the appropriate redox state, and homocitrate, respectively. Finally, the metallocluster carrier proteins NifX, NifY, and NafY aid in FeMo-co synthesis and insertion, but are certainly not essential and could be removed from the equation in initial approaches to engineer novel nitrogen-fixing organisms.

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Chapter 8

Distribution and Ecological Niches of Nitrogenases

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8.1 INTRODUCTION

Biological nitrogen fixation (BNF) is the purview of a single family of enzymes, nitrogenases, with three closely related subclasses of proteins. To date, all nitrogen fixation, as defined by dinitrogen gas (N_2) reduction to ammonia, is limited to this family of nitrogenases found in the diverse Archaea and bacteria. The three variants of nitrogenase are expressed from three gene clusters and are distinguished by characteristic amino acid sequence differences and metal composition (see Chapter 2). This chapter explores the distribution and ecological niches of the organisms equipped with two or all three of these enzymes.

Dinitrogen reduction by all three types of nitrogenase is performed by a two-protein component system requiring a metabolic source of electrons, for example, reduced ferredoxin or flavodoxin, and ATP. The reaction can be summarized by the general equation (Rees and Howard, 2000):

$$N_2 + (6+2n)H^+ + (6+2n)e^- + p(6+2n)ATP \rightarrow 2NH_3 + nH_2 + p(6+2n)ADP + p(6+2n)Pi$$

It is not our intention here to evaluate the mechanistic nuances of the reaction but to emphasize the generality of the overall reaction stoichiometry as discussed in sections below. The present understanding of the enzyme mechanism is that at least one equivalent of H_2 is produced for each mole of N_2 reduced; however, depending on other conditions of the reaction, such as enzyme component ratio and concentration, the ratio of H_2/ammonia can be significantly higher. Likewise, the ratio of ATP to electrons in products can vary from 1 to 50 in uncoupled reactions depending on a number of parameters (Rees and Howard, 2000).

The general structure and organization of the nitrogenases from all three classes are the same. Nitrogenase is composed of Component 1 and Component 2, which are generic terms encompassing all three classes. Component 1 has two different, but similar subunits, designated α and β, which are expressed from the genes *D* and *K* respectively. Two copies of these two subunits are paired to give a tetramer $(\alpha\text{–}\beta)_2$. Shared between the subunits in each α–β pair is the 8Fe:7S P-cluster, while the substrate reduction site in the α subunit is the 7Fe:M:9S:C:homocitrate cofactor shown in Figure 8.1 (see also Chapter 2).

The metal M can be Mo, V, or another Fe atom. It is this difference in metal composition, M (Mo, V, or Fe) at a single site in the cofactor that distinguishes the three cofactors.

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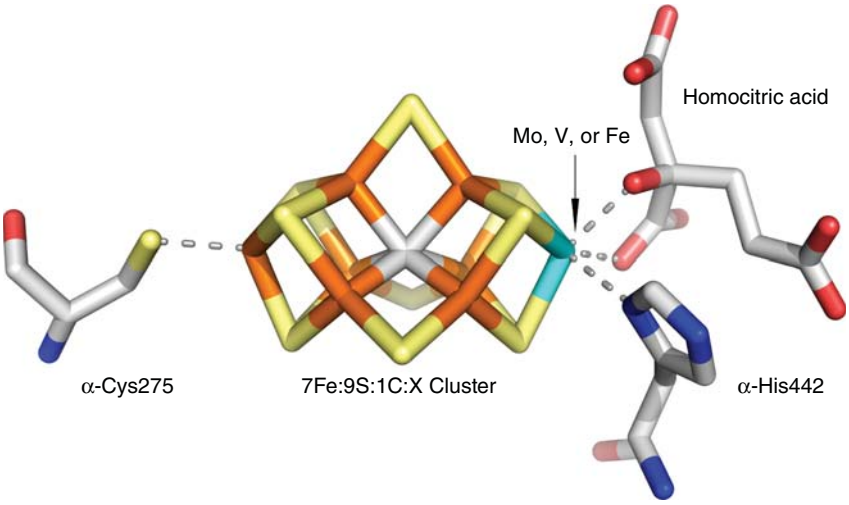

Figure 8.1 Stick figure for the nitrogenase cofactor with protein ligands, α-Cys275 and α-His442. The cofactor includes the organic acid, homocitric acid, and the metallic cluster where X (shown in cyan) = Mo, V, or Fe, depending on the nitrogenase type. The cluster contains the interstitial carbon atom (shown as the gray central atom), hexa-coordinated to six iron atoms (orange). Sulfur atoms are shown in yellow, oxygen atoms in red, and nitrogen atoms in blue. Figure 8.1 uses 1.0 Å coordinates (Protein Data Bank pdb3u7q) (Spatzel et al., 2011; Howard and Rees, 2006; Einsle et al., 2002).

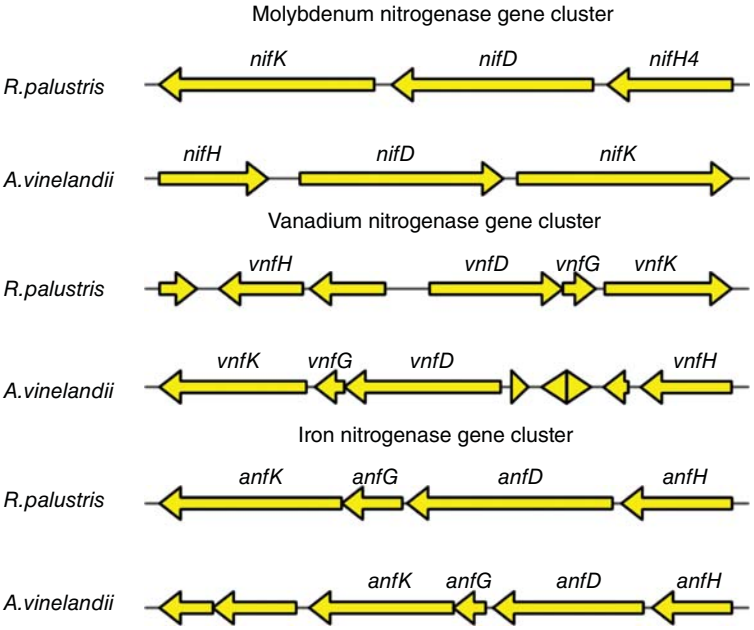

Figure 8.2 Diagrammatic representation of the arrangement of the genes encoding the subunits of Components 1 and 2 of the MoFe-, VFe-, and FeFe-nitrogenases on the chromosomes of *Rhodopseudomonas palustris* (Oda et al., 2005) and *Azotobacter vinelandii* (Setubal et al., 2009).

Associated with each cofactor type is a specific Component 1 with homologous α and β subunits, expressed from the appropriate genes *D* (α-subunit) and *K* (β-subunit) for each of the three classes. The genes are designated *nifD* and *K* for the Mo-containing cofactor, *vnfD* and *K* for the V-containing cofactor, and *anfD* and *K* for the all Fe-containing cofactor. These Component 1 proteins with their unique cofactor and subunits are identified as MoFe-protein, VFe-protein, or FeFe-protein. The Anf and Vnf classes also have a small subunit, designated δ (encoded by *anfG* or *vnfG*), whose function appears to be to stabilize the tetramer, a condition apparently not needed for the Nif class. The number of δ in the tetramer has been uncertain until recently where four were found for the VFe-protein (Lee et al., 2009).

The second component, the generic Component 2, is the Fe-protein having two identical subunits bridged by a single 4Fe:4S cluster (Hausinger and Howard, 1983; see also Chapter 2). The Fe-protein binds two ATPs (one in each subunit) and is the sole functional electron donor to the Component 1 in nitrogenase turnover. Each class of nitrogenase based on the cofactor has its own highly homologous Fe-protein expressed from the gene *H*, or *nifH*, *vnfH*, and *anfH* (Fig. 8.2). For electron transfer, the Fe-protein with two ATPs binds to each α–β pair in Component 1, to form a transient complex where ATP is hydrolyzed. Based upon the accounting for an active site with one Fe-protein bound to one α–β pair, there are at least 19 Fe required, or, for the full tetramer, 38 Fe.

A fundamental question arises from the observation that there are three highly similar classes of nitrogenase, differing primarily in the identity of the single metal at the active site cofactor – what is the survival advantage to having more than one type of nitrogenase? We explore here the variation of the distribution of the classes among diazotrophic species and their ecological niches.

8.2 ALTERNATE NITROGENASES AND THE METAL CONTENT OF THE COFACTOR

The connection between the metal content of the cofactor and the gene of origin, as indicated above, is based upon a limited number of isolated and characterized nitrogenases. The exploration of the general identity of nitrogenases, and of their phylogenetic relationships, relies entirely on the detection of the *nif*, *vnf*, or *anf* genes. Because all diazotrophic species whose genes have been studied contain *nif*, and many fewer contain *anf* or *vnf*, the latter two have been designated as "alternate" nitrogenases (see Chapter 2).

A widely accepted and reasonable assumption is that the expression of these genes is responsive to the metal availability, and, that in consequence each expressed nitrogenase binds a cofactor with the "matching" metal, that is, Mo, V, or Fe, as appropriate. This assumption has been shown to be valid in several diazotrophs, most prominently for *Azotobacter vinelandii* (Joerger et al. 1989, Jacobitz and Bishop 1992, Eady 1996, Premakumar et al. 1998, Howard and Rees, 2006), which under appropriate conditions expresses the three nitrogenases, each equipped with a cofactor with the expected metal composition. In the absence of analytical data on the cofactor for the great majority of nitrogenases, this expectation is assumed to be valid, and that is the case for the discussion presented in this chapter as well. This is a "working hypothesis," whose generality is untested. There are observations in the literature that raise doubts about its general validity (e.g., Pau et al., 1993; Eady, 1996). Where feasible, the assumption needs to be tested on a case-by-case basis by direct chemical analysis of the isolated proteins (Howard et al., 2013).

8.3 DISTINGUISHING FeFe-NITROGENASE FROM VFe-NITROGENASE

Critical to evaluating potential implications of nitrogenase classes and their distribution across ecological niches is to unambiguously identify these proteins in the various diazotrophs. For the most part, the identification depends on the interpretation of a partial or complete genomic sequence determination. Anf and Vnf are homologous proteins with a high percentage of amino acid residue identity. This raises the possibility of confusion in gene identification.

With the rapidly growing annotated databases of complete genomes of diazotrophs, the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/blastcgihelp.shtml) provides a rapid way to survey a wide range of organisms for the presence of alternate nitrogenases. For a protein of interest, BLAST uses a representative amino acid sequence to find and identify related sequences, and provides the statistical data for each of the matches. For nitrogenases, the long-studied *A. vinelandii* Nif, Vnf, and Anf proteins can serve as an excellent source of reference sequences. How well does the BLAST analysis perform in distinguishing AnfD from VnfD, or VnfD from NifD?

As described recently in detail (Howard et al., 2013), comparative analyses of nitrogenase sequences show that the sequences of the NifD, AnfD, and VnfD subfamilies, as well as those of the corresponding K subfamilies, can be unambiguously distinguished by their unique strong motif residues. A strong motif residue is defined as an amino acid found at a sequence-specific site in all members of a subfamily, but absent from that position in members of all other subfamilies (Bickel et al., 2002).

In a BLAST search for similarity to the *Enterobacter radicincitans* DSM 16656 α-subunit, AnfD (EJI90485.1), at least six proteins were incorrectly identified as AnfD (Table 8.1b). These sequences were observed to have high bit scores and >57% identity with the query sequence; these indices would seem to qualify them as authentic Anf group proteins. However, when evaluated by multiple sequence alignment and by strong motif residues, these proteins were clearly not Anf, and indeed, were Vnf (Table 8.1a). The subsets of residues from the presently recognized strong motifs for Anf and Vnf (Howard et al., 2013) were chosen to include residues over the full length of the proteins. The subsets included both overlapping residues (residue position that was a constituent of a strong motif in both Anf and Vnf, yet by definition of strong motifs, a different residue) and residue positions that were uniquely a strong motif for one of these two nitrogenase subfamilies. For the six proteins shown in Table 8.1b, none of the ten-member Anf strong motif residues were found, and all seven residues of the Vnf strong motif subset were present. This clearly places these proteins as Vnf. The latter sequences were interspersed in the search with several Vnf and Nif proteins, correctly identified, with equally compelling bit and percentage identity scores. In all, 48 sequences in this BLAST search were designated as AnfD. As discussed above, six of these were actually VnfD – a 12.5% error rate. These errors substantially skew the ratio of Anf/Vnf sequences. The results clearly demonstrate the care required to ensure the appropriate attribution of a protein sequence to a genotype.

Table 8.1 (a) Strong motif residues in AnfD and VnfD subfamilies. (b) Sequences identified in the BLAST analysis as AnfD, but which have none of the strong motif residues characteristic of AnfD sequences, but all the strong motif residues characteristic of VnfD sequences.

(a)

Residue # Anf sequence	35	36	37	57	87	235	257	345	376	409	433
Residue # Av1 sequence	48	49	50	69	99	252	274	364	394	427	451
Anf residue	G	Y	L	H	I		E	A	C	P	K
Vnf residue				L		H	N	T	G	V	M

(b)

Species	ID	Bit score	% Identical
Methanosarcina acetivorans C2A	NP_616155.1	547	58
Fisherella musicola	WP_016867598.1	551	59
Fisherella sp. *PCC9339*	WP_017308098.1	547	60
Desulfobacter curvatus	WP_020587162.1	536	60
Methylocystis parvus	WP_016920361.1	533	59
Clostridium arbusti	WP_010240510.1	528	57

8.4 WHY THREE NITROGENASES?

All nitrogen-fixing organisms that have been examined have genes encoding MoFe-nitrogenase. How do the two alternative nitrogenases contribute? In terms of total population size, the number of nitrogen fixers equipped solely with MoFe-nitrogenase outnumbers those that also contain alternate nitrogenases by orders of magnitude. There is no reported instance of a nitrogen fixer that contains solely VFe- or FeFe-nitrogenase, but lacks MoFe-nitrogenase. There are only a few microorganisms that have genes for all three nitrogenases. To date, the latter organisms are *A. vinelandii*, *Azotobacter paspali*, *Methanosarcina acetivorans*, and *Rhodopseudomonas palustris* (Bothe et al., 2010), a very short list. Of these organisms, *R. palustris* and the closely related *Rhodopseudomonas* strains have been the most broadly investigated and provide a useful case study of the lessons to be learned from the characterization of very closely related strains isolated from carefully examined sources, and subsequent attempts to explain or predict their ecological niches.

8.5 RHODOPSEUDOMONAS PALUSTRIS CGA009 AND CLOSELY RELATED STRAINS

A subject of study since the early years of the twentieth century, *R. palustris* CGA009 (van Niel, 1944) is famed for its exceptional metabolic versatility (Wall, 2004; Larimer et al., 2004). Sequencing of the *R. palustris* CGA009 genome revealed a full suite of genes for all three metallonitrogenases. *R. palustris* CGA009 is unique among purple phototrophic bacteria in having a VFe-nitrogenase (Larimer et al., 2004).

Complete genome sequences have been determined for four closely related *Rhodopseudomonas* strains: BisB18, Bis5, BisA53, and HaA2 (Oda et al., 2008). The sequence of strain TIE-1 was also available (Jiao et al., 2005). Table 8.2 describes the sources of these strains.

The 16s rRNA sequence identity between CGA009 and the five strains ranged from 100% for TIE-1 to 97.8 to 97.3% for the other four. Average percent amino acid identities between pairs of orthologs of the *Rhodopseudomonas* genomes for CGA-009, BisB18, BisB5, and BisA53, and HaA2 ranged from 69.6% (CGA009, BisB18) to 87.0% (HaA2, BisB5), with an overall average of ∼73%.

Whole-genome comparisons in the 1970s by DNA–DNA hybridization showed that members of same-named prokaryote species, in contrast to members of different-named species, share greater than 70% of their genome content (Johnson, 1973). Subsequently, the >70% metric was accepted formally by bacterial systematists (Wayne et al., 1987). Now, a difference of ∼1% in 16S rRNA sequence between strains is viewed as sufficient to assign them to different species (Stackebrandt and Ebers, 2006; see Chapter 3). Since the data provided above shows strain TIE-1 as the only strain to pass the 16S rRNA identity test, the other strains have been designated as *Rhodopseudomonas* spp.

A view in high detail of the similarities and differences between the proteomes of these strains provides insights into the relationship between these organisms and their ecological niches and the way to a more nuanced understanding of the distribution and roles of the nitrogenases.

As shown in Table 8.3, the five strains are clearly strongly related. However, in each strain, 10–18% of the genes are strain specific.

Strain CGA009 encodes MoFe-, FeFe-, and VFe-nitrogenases. Strains BisB18 and BisA53 encode

Table 8.2 *Rhodopseudomonas* sp. strains: Sources and Mo-, W-, and FeFe-Nitrogenase gene representation

Strain	Nitrogenase Genes	Source of Strain
CGA009	Mo, V, Fe	CGA009 is a chloramphenicol resistant derivative of CGA 001, from the culture collection of R.K. Clayton at Cornell University.*
TIE-1	Mo, Fe	Isolated from cultures of phototrophic Fe(II)-oxidizing bacteria enriched in a medium supplemented with 10 mM $FeCl_2$ inoculated with samples taken from an iron-rich mat from School Street Marsh in Woods Hole, MA.
HaA2	Mo	HaA2 came from a site roughly ~240 km from the first two locations, from a 1– to 2-mm-thick patch of leaf litter, roots, and sediment present ~2 cm below the surface of a shallow pond that was formed by the accumulation of rainwater in a depression.
BisB18 BisB5 BisA53	Mo, Fe Mo Mo, Fe	These strains were isolated from the top 0.5 cm of claylike sediment present 1–2 cm below the surface of a river along its bank. Strains BisB18 and BisB5 were isolated from the same 0.5 g of sediment sample. BisA53 was from a sample taken about 5 m away.

*Kim M-K; Harwood CS (1991); Oda et al., 2008.

Table 8.3 Comparative gene inventories of five strains of *Rhodopseudomonas*

Strain	Total Number of Genes	Strain-Specific Genes*	Genes Shared by all Five Genomes*
CGA009	4833	585	2752
HaA2	4683	514	2740
BisB18	4886	859	2751
BisB5	4397	420	2746
BisA53	4884	794	2760

*Including paralogs.

Oda et al., 2008; Jiao et al., 2005.

MoFe- and FeFe-nitrogenase, and HaA2 and BisB5 encode only MoFe-nitrogenase.

The genomes of TIE-1 and CGA009 are 97.9% identical at the nucleotide level over 5.28 Mb of shared DNA. Notably, TIE-1 encodes only the MoFe- and FeFe-nitrogenases. Comparison of these highly homologous genomes shows that the VFe-nitrogenase gene cluster in CGA009 is part of an indel that is not present in the TIE-1 genome sequence, strongly indicating that the V-nitrogenase was acquired by lateral gene transfer, a deduction supported by its absence from the other *Rhodopseudomonas* strains (Oda et al., 2008), and more broadly, from purple phototrophic bacteria.

A comparison of the available data on the attributes of the environment from which each of the *Rhodopseudomonas* sp. strains was isolated (Table 8.2) and of the nitrogenase genes in each of the organisms strongly suggests that the attempt to predict the microenvironment from which one is sure to isolate a particular nitrogenase genotype would be unproductive. Oda et al. (2008) comment that "the *Rhodopseudomonas* isolates are different ecotypes that evolved by radiating into microenvironments with distinctive characteristics of light, oxygen, and nutrient availability while retaining a high degree of metabolic flexibility" and conclude that "Although the microenvironments from which each of these *Rhodopseudomonas* ecotypes derive are on a scale that may be too small to allow accurate measurement of their physical and chemical characteristics, we can perhaps infer these characteristics from the genome sequences."

Significant problems confront the attempt to describe a microenvironment as the environment "preferred" by a *Rhodopseudomonas* ecotype. *Rhodopseudomonas* spp. are motile. The genome of each of the six strains, listed in Table 8.2, encodes flagellin genes. It is likely that these organisms explore multiple microenvironments and, because of the great breadth of their metabolic capabilities, may do equally well under significantly different conditions. Moreover, the substantial number of genes specific to each strain suggests that the microenvironment "ideal" for each strain

may be distinctive, but the inspection of its genome is very unlikely to allow an *a priori* exclusive definition of that microenvironment.

8.6 CONTROL OF EXPRESSION OF NITROGENASE

Two linked questions need to be considered from the perspective of natural selection: what is the survival advantage of carrying multiple forms of nitrogenase, and if there is such an advantage, why is *nif* the sole nitrogenase in the great majority of diazotrophic species? To gain a glimpse of the answers one needs to start by considering when and how the nitrogenases are expressed. Understanding what controls the *in vivo* expression of each of the three nitrogenases is an essential part of understanding the relationship between a particular species and its ecological niche(s). An important caveat needs to be kept in mind while seeking answers: what we know, even when the knowledge is limited, is for a relatively small sample of the nitrogen-fixing species and generally those from less demanding environments. Fortunately, the number of diverse organisms under study and the variety of available experimental approaches are rapidly expanding, and enhance our ability to explore multiple fundamental questions.

Our present understanding of relevant regulatory processes in diazotrophs is that the intracellular metal composition and nitrogen compounds (principally ammonium ion and glutamine) independently regulate the level and class of nitrogenase expressed. The intracellular levels of both of these types of regulatory agents are indirectly dependent upon their transport from the environment. A full discussion of the role of nitrogenous compounds in regulating nitrogenase expression is outside the scope of this chapter and is addressed elsewhere (Dixon and Kahn, 2004). Understanding of the molecular basis of metal control for several species (Hamilton et al., 2011, Heiniger et al., 2012) is improving. Research on diazotrophs in a controlled laboratory environment found uniformly that the metal-dependent classes of nitrogenase were expressed in order of preference for Mo over V, and of Mo or V over Fe. When Mo was available, diazotropic growth and transcription analyses under nitrogen-fixing conditions showed that *nif* genes were expressed while neither *vnf* nor *anf* genes were expressed.

However, this generalization of the metal control of gene hierarchy appears to be much more complex when analyzed in detail in some species. For example, the mutant strain *R. palustris* ΔnifH nifD::Tn5 (strain with *nif* structural genes deleted or disrupted) expressed active FeFe- and VFe-proteins when grown diazotrophically in the presence of 15 nM molybdate salt; even the addition of Mo to concentrations as high as 100 μM did not substantially affect the growth rates, or rates of acetylene reduction when only the FeFe- or only the VFe-nitrogenase were expressed (Oda et al. 2005). In contrast, a *Rhodobacter capsulatus* ΔnifHDK mutant strain, similar to the *R. palustris* mutant strain, was unable to express its *anf* genes or grow diazotrophically at Mo concentrations of >10 nM (Schneider et al., 1991). Certainly there are species differences beyond just the nitrogenase genes at work here. It is well to note that in most, if not all, of these genetically manipulated strains, the actual proteins and their metal content have not been examined. It is evident that when the *nif* genes are deleted, no structural MoFe-protein is expressed. However, the metal content of the cofactor incorporated by the structural proteins expressed from *vnf* or *anf* genes in the presence of Mo has not always been determined (Oda et al., 2005). In at least one case, *A. vinelandii*, if the *nif* genes were deleted but the cells grown with Mo in the medium, the Mo form of the cofactor is inserted into the FeFe-protein and diazotrophic growth is enabled (Pau et al., 1993). In another species, *R. capsulatus*, tungsten will substitute for Mo in the cofactor although the resulting nitrogenase is only able to reduce protons to H_2 but not N_2 to ammonia (Siemann et al., 2003). These examples show that there may be surprises in store regarding which metals are used and which gene of origin, *nif*, *anf*, or *vnf*, is expressed to construct an active nitrogenase.

Biochemical studies on the reactions catalyzed by MoFe-protein, VFe-protein, and FeFe-protein and their associated Fe-proteins, each purified and with the metal defined, suggest differences in the relative ratio of H_2 and ammonia during turnover as well as in ATP used per electron transferred (Eady, 1996, 2003); also see the equation in the Section 8.1.

It is tempting to extrapolate these differences to suggest advantages of one enzyme class over another in terms of preferred ecological niches. For example, it has been suggested that the VFe-protein might be a more useful enzyme for an organism growing at 4°C as it retains a higher dinitrogen reduction fraction of activity than does the MoFe-protein at that temperature (Miller and Eady, 1988). As important as such studies are for understanding the enzymatic mechanism, they fall short of answering questions about the rationale of the ecological distribution of nitrogen fixation. As noted earlier, the alternate proteins are poorly characterized in terms of quaternary structure and the role or the stoichiometry of the δ subunit. Many of the conditions critical for exploring the relative efficiency of the three enzymes have not been reproduced, casting doubts on the reliability of such comparisons.

It is worthwhile enumerating the differences between *in vitro* measurements and nitrogen fixation *in vivo*.

1. The concentration of the proteins is orders of magnitude higher in a cell where ca. 5–10% of the cellular mass is represented by the nitrogenase proteins (Dingler et al., 1988; Jacobs et al., 1995). Because the complex between the Fe-protein and Component 1 is central to the overall

reaction, the effects of concentration are large and, perhaps surprisingly, catalysis is slower on a molar turnover basis at the high protein concentrations found in the cell.

2. The ratio of Fe-protein to Component 1 is near unity in the cell, while in *in vitro* studies the maximum activity is achieved at a higher ratio (Dingler et al., 1988; Jacobs et al., 1995). Again, the *in vivo* activity is lower at such component ratios.
3. *In vivo*, the penultimate electron donor (donor for the Fe-protein) is a flavodoxin or ferredoxin (connected to other cellular metabolism) that can reduce the Fe-protein by two electrons. *In vitro*, dithionite, a poorer reducing agent with many side products, is used and is only capable of reducing the Fe-protein by one electron. Hence, ATP hydrolysis in the *in vitro* assay might be twice the actual biological requirement; see equation Section 8.1.
4. ATP and a regenerating system are used *in vitro* to set the ADP/ATP ratio near zero, while *in vivo*, the cellular "energy charge" is set by the overall metabolism, and where ADP, a known inhibitor of nitrogenase, is present in substantial amounts (Upchurch and Mortenson, 1980). The "energy charge" is certainly a function of metabolic sources and must be a significant variable between niches, for example, phototrophs *versus* chemotrophs.

The intracellular concentrations of nitrogenase represent a marked difference between the conditions under which nitrogen fixation proceeds *in vivo* and those used to study the enzyme mechanism *in vitro*. Indeed, a projected efficiency based on the *in vitro* numbers would suggest that MoFe-nitrogenase was much preferred over the two alternate nitrogenases, yet when strains of *Azotobacter* were analyzed by growth rates in chemostats, the FeFe-protein system was essentially equal to the MoFe-protein (Bishop et al., 1986). More recent studies have shown that in *A. vinelandii*, transcription of the three metal classes of nitrogenase are different, possibly leading to different levels of cellular nitrogenase, and furthermore, the utilization of nitrogenous compounds is affected when cells shift to an alternate nitrogenase (Hamilton et al., 2011). Similar changes are seen in *R. palustris* along with more efficient uptake of extracellular ammonia (Oda et al., 2005).

These results clearly show the need for studies that more closely mimic the various ecological niches, such as the actual metal concentrations and their oxidation states, as well as energy sources, and the pH in the immediate environment. Evaluation of how the varied expression of the three nitrogenases provides "fitness" for an ecological niche and evolutionary survival would be greatly aided by determining growth rates in a chemostat under conditions simulating those of the potential niche.

8.7 GLOBAL DIVERSITY OF NITROGEN-FIXING ORGANISMS

It is plausible to assert that BNF is performed on Earth wherever life is found, as exemplified by the presence of diazotrophs in the Arctic tundra (Davey, 1982), the abyssopelagic zones of the Sargasso Sea (Hewson et al., 2007), and at the extreme temperatures of the sea hydrothermal vent fluids (Mehta and Baross, 2006). A caveat is that frequently little is known about the gene expression patterns of the diazotrophs in the various environments (Riemann et al., 2010), or even about the frequency of representation of Vnf and Anf relative to Nif.

From extensive studies of *nifH* phylotypes, Zehr et al. (2003) concluded that the distribution of nitrogen-fixing organisms, although strongly influenced by fixed nitrogen availability in the environment, is nonrandom and can be predicted on the basis of habitat characteristics. The phylotypes fall into four clusters (Chien and Zinder, 1996; Zehr et al., 2003). Cluster I is composed of bacterial *nifH* and some *vnfH* sequences. Cluster II contains bacterial and methanogenic (Archaeal) *anfH* sequences. Cluster III contains *nifH* sequences from anaerobic members of the Bacteria and Archaea. Cluster IV contains *nifH* paralogues that have no role in nitrogen fixation.

Gaby and Buckley (2011) utilized an aligned database of 16,989 *nifH* sequences, then available in public databases, to assess the diversity of diazotrophs across phylogenetic groups or across environments. Using a DNA barcode approach and clustering these sequences into molecular operational taxonomic units (OTUs; e.g., (Blaxter et al., 2005; Hao et al., 2012)), this study provided a glimpse of the diazotroph diversity in different environments and in different *nifH* lineages. Of the 16,989 sequences, 13% fell into Cluster III.

Soil, marine sources, microbial mats, and termite guts – ranked in order of the number of *nifH* sequences derived from the source environment – accounted for 64% of the sequences, with soil being by far the richest. Sequences from α, β, and γ *Proteobacteria* and from *Cyanobacteria* represented 46% and 30%, respectively, of the sequences from marine environment. Gaby and Buckley (2011) noted that these data are strongly influenced by site selection in the surveys of microbial diversity, and consequently should not be taken as a measure of global abundance. Rather, they noted, "they provide information on nitrogen-fixing organisms most commonly observed in sequence databases." Of the ten most observed taxa, five are from *Cyanobacteria*, and the other five from α, β, and γ *Proteobacteria*. Strikingly, five of the 10 top OTUs did not contain cultivated representatives. However, a detailed discussion of the results of this study provides valuable insights on the correlation between physiological and biochemical properties of diverse diazotrophs and the environments in which they are found

(Gaby and Buckley, 2011). Notably, this analysis did not include much information on Vnf or Anf nitrogenases. Apparently, it is difficult to resolve *nifH and vnfH* in some instances (Zehr et al., 2003).

Fortunately, some information pertaining to the distribution and phylogeny of organisms with genes for VFe- and FeFe-nitrogenases is available from other specific, focused studies, essentially "case histories."

8.8 ISOLATION OF DIAZOTROPHS CONTAINING Mo-INDEPENDENT NITROGENASES

Studies on global diversity of diazotrophs leave the impression that nitrogen fixers with alternate nitrogenases represent a very small fraction of such organisms. However, the results of studies employing standard microbiological methods of enrichment and cultivation in pure culture indicate that organisms with VFe- and/or FeFe-nitrogenases are present in many different aquatic and terrestrial environments.

Bishop and coworkers (Loveless et al., 1999; Betancourt et al., 2008) successfully isolated, by enrichment, pure strains of such organisms from a wide range of natural environments, including creek sediment, mangrove sediment, salt marsh, soil, "paraffin dirt" (Simoneit and Didyk, 1978), wood chip mulch, and so on. The alternate nitrogenases in each of these strains were identified by PCR amplification using either *vnfDGK* or *anfDGK* primers. The 16S rRNA analysis revealed that some three-quarters of these strains belonged within groups of long-studied γ-proteobacterial diazotrophs: *A. vinelandii*, *Azobacter beijerinckii*, and *Azomonas agilis.* In the study by Loveless et al. (1999), seven of the γ-proteobacterial diazotrophs, isolated from a variety of habitats, had the genes for MoFe-nitrogenase and VFe-nitrogenase, and grew well on V_2O_5 under Mo-deficient, nitrogen-fixing conditions, although with generation rates 20–30% slower than those seen in the presence of 1 mM Na_2MoO_4 or 10 mM NH_4^+. Four of these isolates also had the genes encoding FeFe-nitrogenase.

8.9 COINCIDENTALLY DISCOVERED NOVEL GENERA AND SPECIES WITH ALTERNATE NITROGENASES

Pelosinus fermentans (type strain $R7^T$), the first representative of a new genus (Shelobolina et al., 2007; Yutin and Galperin, 2013), was isolated from primary kaolin, a clay from an alluvial deposit in Southern Urals in Russia. Kaolin is a highly prized source of material for porcelain manufacture. Deposits of kaolin have also been found in China, England, and the United States. Kaolin contains (by weight) 0.25–1.5% Fe_2O_3 (Jepson, 1984). Climatic conditions favorable for weathering lead to removal of discoloring impurities, such as iron oxides and sulfides, and organic matter, and result in white kaolin (Elzea Kogel et al., 2002). These observations led to a project to isolate representative Fe(III)-reducing bacteria from kaolin clays that may play a role in the removal of the iron from kaolin. This effort led to the isolation, under strict anaerobic conditions, of an isolate, strain R7, that was capable of reducing Fe(III) only in the presence of a fermentable substrate (Shelobolina et al., 2007). Based on phylogenetic analysis and physiological tests, this strain was proposed to represent a new genus, *P. fermentans* gen. nov., sp. nov., type strain R7. The complete genome of strain R7 is available (Brown et al., 2012.), and it has the genes encoding MoFe- and FeFe-nitrogenases.

There is long-standing interest in bioremediation of uranium-contaminated waters by a wide variety of bacteria capable of reducing U(VI) to U(IV) (Wall and Krumholz, 2006). In oxic waters and soils, uranium is present as soluble uranyl ion (UO_2^{2+}) in the oxidation state U(VI). Reduction of U(VI) to U(IV) produces the insoluble mineral uraninite and thus the sequestration of uranium (Langmuir, 1978). The same opportunity for intervention is presented by Cr(VI), toxic, and mobile in groundwater. When reduced to the Cr(III) state, the metal complexes are much less toxic and form insoluble, stable precipitates under slightly acidic to neutral conditions in aquifers (Palmer and Wittbrodt, 1991; Cook, 2000).

To enrich for bacteria capable of reducing Cr(IV), contaminated groundwater from Hanford, Washington (USA), was amended with 30 mM lactate and 0.05 mM sulfate, in a reactor kept anaerobic with N_2 gas. Under these conditions of electron acceptor limitation, the enrichment led to a predominance of metal-reducing *Pelosinus* spp. (De León et al., 2012; Mosher et al., 2012). Four of the *Pelosinus* spp. strains (A11, A12, B3, and B4) were obtained in pure culture, their genomes were sequenced and found to be very similar, and each was tested for the ability to reduce Fe(III), monochromate, dichromate, and U(VI). *P. fermentans* strain A11 reduced all four, strain R7 (isolated from kaolin) and strain B3 were able to reduce Fe(III), monochromate, dichromate, but not U(VI), strain A12 only Fe(III), strain B3 Fe(III) and monochromate (Table I in Mosher et al., 2012). As far as nitrogenases are concerned, strain R7 has the genes for both MoFe- and FeFe-nitrogenases (genome data from Brown et al., (2012.)), as do all four Hanford strains.

8.10 ESTIMATES OF CURRENT GLOBAL NITROGEN FIXATION

Annual global nitrogen fixation, including an anthopogenic contribution of 210 Tg N/year, is estimated at 413 Tg N/year.

The subtotals (in Tg N/year) that make up these totals have large error bars (Fowler et al., 2013; Vitousek et al., 2013). The estimate for non-agricultural BNF on land is $58 \pm 50\%$ (i.e, between 29 to 87 Tg N/year) to which agricultural BNF adds $60 \pm 30\%$. Fertilizer production contributes $120 \pm 30\%$, combustion $30 \pm 10\%$, and lighting $5 \pm 50\%$. BNF in marine ecosystems contributes $140 \pm 50\%$ (Fowler et al., 2013).

In terrestrial systems, it is difficult to determine with precision the quantitative contribution made by BNF. BNF is broadly divided into symbiotic and free living. This division obscures the diversity of relationships between nitrogen-fixing microorganisms and plants. Reed and Cleveland (2011) provide a more nuanced description, but one that still adheres to this subdivision: "… we define symbiotic N_2 fixation as N_2 fixation that occurs via relationships between plants (e.g., legumes) and the N_2-fixing microbial symbionts occupying plant root nodules (e.g., *Rhizobia* or *Frankia*). In contrast, we classify all other forms of N_2 fixation (including N_2 fixation by epiphytes on plant leaf surfaces and the symbiotic N_2 fixation that occurs in lichens) as free-living N_2 fixation" (Reed and Cleveland, 2011). They also comment that the commonality and activity of alternative nitrogenases remains unknown.

In some instances, the presence of an alternative nitrogenase may be known, but goes without comment. *E. radicincitans* DSM16656^T, a proteobacterium now reclassified as *Kosakonia radicincitans* DSM16656^T (Brady et al., 2013), was isolated from the phyllosphere of winter wheat under temperate climatic conditions. Upon the inoculation of various plant species, this organism was reported to promote the growth of roots and shoots and increased yields (Remus et al., 2000). Its genome has genes for nitrogen fixation, phosphorus mobilization, and phytohormone production (Witzel et al., 2012). Nitrogenase genes for both MoFe-nitrogenase and FeFe-nitrogenase are present. Given the ease of detection of genes for VFe- and FeFe-nitrogenase, doubtless, over time a clearer picture will emerge of their distribution.

8.11 CONCLUDING REMARKS

In 1913, the Haber–Bosch process provided the first useful industrial process for reduction of N_2 to ammonia, a highly energy-demanding reaction requiring 200–500 atm at 500–600°C with an iron-based catalyst. Exactly one hundred years later, this process, now improved with a ruthenium-based catalyst, still requires high pressure and temperature (70–80 atm, 350–470°C) (Tanabe and Nishibayashi, 2013). By contrast, for the past 2 billion years, the microbial world has performed the synthesis of NH_3 from N_2 and H_2 at less than 1 atm and 25°C.

Demand for fixed nitrogen continues to rise, with current commercial ammonia production reaching 160 million tons per year. As a reference point, the mass of N_2 in the atmosphere is 3.9×10^9 Tg. There is no danger of running out of raw material.

"Human activity has greatly increased the rate of transfer of N_2 to the biosphere (industrial manufacture of fertilizer, fossil fuel combustion, nitrogen-fixing crops), resulting possibly in a global fertilization of the biosphere" (Jacob, 1999). With respect to symbiotic microorganisms, the rate of diversity decline among host organisms is accelerating in response to climate change. It is inevitable that the increasing inputs of nitrogen fertilizers, along with the impacts of climate change, will affect the global distribution and diversity of nitrogen-fixing prokaryotes, and the ongoing surveys of microbial diversity will serve as "shifting baselines" for future ecological studies.

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Section 3

Expression and Regulation of Nitrogen Fixation Genes and Nitrogenase

Chapter 9

Regulation of *nif* Gene Expression in *Azotobacter vinelandii*

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9.1 INTRODUCTION: *Azotobacter vinelandii* AS PARADIGM OF NITROGEN-FIXING BACTERIA

Only some bacteria and archaea are capable of performing biological nitrogen fixation. Organisms that grow on N_2 as sole nitrogen source are called diazotrophs, and play an essential role in the biosphere (Falkowski, 1997; Boyd et al., 2011; see Chapter 1). Most biological nitrogen fixation is catalyzed by the Mo nitrogenase, although some nitrogen-fixing bacteria additionally contain alternative V or Fe-only nitrogenases that are expressed when Mo is not available in the medium (Bishop and Joerger, 1990). Diazotrophs use large amounts of ATP to break the N_2 triple bond. The Mo nitrogenase requires a minimum of 16 molecules of ATP to fix one molecule of N_2 according to the following reaction (Seefeldt et al., 2009):

$$N_2 + 8e^- + 16\text{MgATP} + 8H^+ \rightarrow 2NH_3 + H_2 + 16\text{MgADP} + 16\text{Pi}$$

It should be noted that H_2 is an obligate subproduct of this reaction, thus decreasing the energetic efficiency of N_2 fixation (Simpson and Burris, 1984). Alternative nitrogenases direct more electrons to the reduction of H^+ than the Mo nitrogenase and, consequently, require the hydrolysis of 24–32 ATP molecules per N_2 fixed (Eady, 1996). All nitrogenases are slow enzymes because they must carry out a minimum of eight electron transfer events, each one occurring at a rate of $5s^{-1}$ (Thorneley and Lowe, 1983). As a result, N_2-fixing bacteria are required to produce large amounts of nitrogenase – up to 10% of the total protein content in the cell – to be competent enough for diazotrophic growth (Dingler et al., 1988). Moreover, the assembly of nitrogenase metal cofactors requires complex biosynthetic pathways involving a number of gene products (Rubio and Ludden, 2008; Hu and Ribbe, 2011; see Chapter 7), some of which must be recycled frequently (Martinez-Noel et al., 2011). It is probably due to these reasons that diazotrophic growth occurs only in narrow nutritional and physiological conditions (Postgate, 1998).

Owing to the extreme oxygen-labile nature of nitrogenase, most diazotrophic bacteria fix N_2 only when growing anaerobically. However, this is not the case of *Azotobacter vinelandii*, a strict aerobic bacterium able to grow diazotrophically almost as fast as when using NH_4^+. *A. vinelandii*, a gram-negative Pseudomonadaceae, has emerged as the leading model for free-living diazotrophic bacteria. There are good reasons for this choice: the convenience of its strictly aerobic metabolism (Burk, 1930) to set up laboratory cultures; the ability to grow of many carbon sources including sugars, alcohols, and organic acids (Wong and Maier, 1985); its genetic tractability (Dos Santos, 2011); and the great quantity and quality of nitrogenase enzymes produced (Dingler et al., 1988).

A. vinelandii ensures an anaerobic intracellular environment by virtually eliminating O_2 traces using highly active respiratory chains (Poole and Hill, 1997; Bertsova et al., 2001). It is also one of the few species carrying all three nitrogenase systems (Bishop and Joerger, 1990). When

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growing under diazotrophic conditions, *A. vinelandii* selects the nitrogenase to express on the basis of metal availability. Priority is on Mo nitrogenase expression, because it is the most efficient in reducing N_2 into NH_3, minimizing H_2 production. In fact, *A. vinelandii* growing diazotrophically in the absence of Mo has been shown to express the urocanate hydratase (*hutU*) gene, which encodes an enzyme involved in amino acid catabolism, suggesting that *in vivo* performance of alternative nitrogenases may not be enough for optimal diazotrophic growth (Hamilton et al., 2011b). The presence of Mo in the medium represses the expression of alternative nitrogenases. In the absence of Mo, and depending on whether V is present in the medium, *A. vinelandii* expresses either the V or the Fe-only nitrogenase (Joerger et al., 1989b, 1990). The fact that *A. vinelandii* can manage the biosyntheses of three different nitrogenase active site cofactors (FeMo-co, FeV-co, and FeFe-co) differing only in one metal site demonstrates high adaptability to grow diazotrophically in poor environments.

9.2 METHODOLOGIES USED TO STUDY REGULATION OF *nif* GENE EXPRESSION IN *A. vinelandii*

Although *A. vinelandii* can accommodate from 4 up to 100 chromosome copies per cell, depending on the stage in the growth cycle (Maldonado et al., 1994), for the most part this bacterium behaves as haploid (Maldonado et al., 1992), allowing easier genetic manipulations. Chromosomal insertions, deletions, and gene replacement techniques are possible because of highly efficient double-recombination events (Dos Santos, 2011). These relatively easy genetic techniques came in handy to determine the *nif* gene map and operon distribution in the *A. vinelandii* genome (Joerger and Bishop, 1988; Jacobson et al., 1989a, b).

Mapping *nif* gene expression was first approached by Northern Blot analysis (Jacobson et al., 1986; Joerger and Bishop, 1988) and *lacZ* transcriptional fusion techniques (Rodriguez-Quinones et al., 1993). Co-transcription patterns within *nif* operons (Jacobson et al., 1986; Hamilton et al., 2011a) and identification of transcriptional regulatory elements (Bennett et al., 1988; Joerger et al., 1989a; Blanco et al., 1993; Walmsley et al., 1994; Drummond et al., 1996) followed. Dixon and collaborators have studied both NifA and NifL biochemistry and genetics extensively (Dixon and Kahn, 2004; Martinez-Argudo et al., 2004b). In addition, considerable efforts have been made to understand the formation of NifA-NifL-GlnK complexes by using protein–protein interaction techniques both *in vivo* (Rudnick et al., 2002) and *in vitro* (Little et al., 2002). More recently, massive sequencing techniques delivered the *A. vinelandii* genome (Setubal et al., 2009) sequence and the transcriptional profiles of *A. vinelandii* grown diazotrophically under different metal availability regimes (Hamilton et al., 2011b).

9.3 *nif* GENES

The synthesis of active Mo nitrogenase is a complex biological process. It involves a number of gene products functioning as nitrogenase component proteins, molecular scaffolds, metallocluster carrier proteins, and additional enzymes, which are required, not just for the synthesis of the nitrogenase complex itself, but also for the biosynthesis of its catalytic metal cofactors (see Chapter 7).

Mo nitrogenase genes in *A. vinelandii* are located in two chromosomal regions adjacent and equidistant from the replication origin (Setubal et al., 2009). From a genetic standpoint, such a position suggests a higher gene dosage during active cell growth (Setubal et al., 2009) or just a critical role of these genes in *A. vinelandii* life style. Interestingly, all genes known to be required for the Mo, V, and Fe-only nitrogenases (e.g., *nifU*, *nifS*, *nifV*, *nifM*, and *nifB*) are located in Mo nitrogenase regions (Joerger and Bishop, 1988; Jacobson et al., 1989a; Kennedy and Dean, 1992; Drummond et al., 1996).

The so-called major *nif* cluster contains at least five transcriptional units, namely *nifHDKTY*, *nifENX*, *orf5*, $iscA^{nif}nifUSVcysE1^{nif}nifWZMclpX2$, and *nifF*, in which *nif* genes are interspersed with a number of open reading frames (ORFs) of unknown function (Jacobson et al., 1989a, b). The minor *nif* cluster contains three operons, namely *rnfABCDGEH*, *nifLA* and *nifB fdxN nifOQ rhdN* $grx5^{nif}$, and the *nafY* gene (Joerger and Bishop, 1988; Rodriguez-Quinones et al., 1993; Rubio et al., 2002; Curatti et al., 2005).

Gene expression in *nif* operons is dependent on the RNA polymerase σ^{54} factor and on the NifA transcriptional activator (Fig. 9.1). The σ^{54} subunit (also known as RpoN, σ^N, or NtrA) recognizes a promoter-specific sequence located at positions −24 to −12 that conforms to the consensus sequence 5′-YTGGCACGR-N_3-TTGCW-3′ (Barrios et al., 1999). In *nif* operons, transcription initiation by the σ^{54}-dependent RNA polymerase requires that the enhancer-binding-protein (EBP) NifA binds to DNA regions known as upstream activator sequences (UAS), which conform to the consensus sequence 5′-TGT-N_{10}-ACA-3′ (Buck et al., 1986). There are cases in which additional DNA-binding proteins are needed, including the integration host factor (IHF) (Hoover et al., 1990). IHF is an asymmetric histone-like protein that binds and bends DNA in specific locations (consensus sequence 5′-WATCAANNNNTTR-3′) (Goosen and van de Putte, 1995).

The *nifHDKTY orf1orf2* operon contains the Mo nitrogenase structural genes *nifH*, *nifD*, and *nifK*, along with *nifT*, *nifY*, and two ORFs of unknown function. Despite being transcribed from the same promoter, transcript levels were up to 70 fold higher for *nifH*, *nifD*, and *nifK* than for the other co-transcribed genes (Hamilton et al., 2011b). Analysis of intergenic sequences within the operon revealed regions

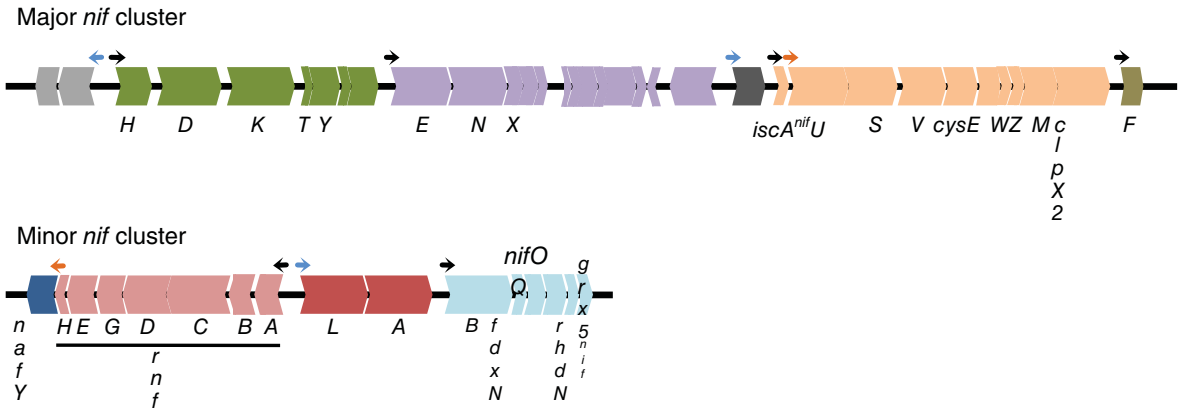

Figure 9.1 *A. vinelandii* Mo-nitrogenase *nif* gene clusters. Predicted σ^{54}–dependent promoter regions are depicted by arrows. Black arrows represent regions additionally containing NifA-UAS and IHF motifs; blue arrows represent regions containing NifA-UAS motifs; orange arrows represent regions lacking both NifA-UAS and IHF motifs.

capable of producing secondary structures that could explain the pattern of mRNA accumulation (Hamilton et al., 2011a). Importantly, overall *nifH* transcript levels also appeared to be higher than *nifDK* levels, revealing a mechanism to control the stoichiometry of nitrogenase component proteins.

The *nifENX orf3orf4* operon appears to have arisen from a duplication of the *nifHDK* operon (Fani et al., 2000). It contains the *nifE* and *nifN* genes, which products are homologous to NifD and NifK, respectively, and form a heterotetrameric $NifE_2N_2$ protein that functions as molecular scaffold for FeMo-co biosynthesis (Brigle et al., 1987; see Chapter 7). In addition, the operon contains the *nifX* gene, which encodes a FeMo-co precursor carrier protein, and two ORFs of unknown function. Although *nifENX* and *nifHDK* operons present similar 5′-untranslated region structures, *nifEN* levels are much lower than those of *nifDK*.

Not much is known about regulation of *orf5* expression except that it is apparently under the control of NifA and its mRNA accumulates in N_2-fixing conditions. However, *orf5* function is not essential for diazotrophic growth (Jacobson et al., 1989a).

The *iscA*^{nif} *nifUSV cysE1*^{nif} *nifWZM clpX2* operon includes a number of genes coding for proteins involved in the biosynthesis of nitrogenase metal cofactors and in the maturation of NifH and NifDK structural polypeptides. Operon expression is turned on once conditions for diazotrophic growth occur (Hamilton et al., 2011b), and its kinetics is similar to other *nif* operons (Poza-Carrión et al., 2014). The products of four genes in this operon (*nifU*, *nifS*, *nifV*, and *nifM*) are required for all three nitrogenases in *A. vinelandii* (Kennedy and Dean, 1992; see Chapter 7). Although *iscA*^{nif} shows the highest expression level in the operon (Hamilton et al., 2011b), its deletion did not impair diazotrophic growth (Jacobson et al., 1989a) probably because other proteins are able to replace its function as scaffold for [Fe–S] cluster biosynthesis. The *cysE1*^{nif} gene encodes a serine *O*-acetyltransferase, an enzyme that catalyzes the first step in L-cysteine biosynthesis; its function is not essential for diazotrophic growth under standard laboratory conditions (Jacobson et al., 1989a). Similarly, *clpX2* was shown to have a role in recycling NifB and NifEN polypeptides (Martinez-Noel et al., 2011) but its function is not essential for diazotrophic growth.

The flavodoxin-encoding gene *nifF* (Bennett et al., 1988) is in an independent transcriptional unit regulated by NifA and located downstream of *clpX2*.

In the minor *nif* cluster, the *rnfABCDGEH* operon encodes for a membrane-bound NADH oxidase complex. Mutational analysis suggested that *rnf* gene products were part of a redox regulatory mechanism controlling the rate of *nif* expression, probably via reduction of NifL (Curatti et al., 2005). In addition, *rnf* mutants exhibited lower rates of incorporation of the [4Fe–4S] cluster into NifH. Importantly, *rnf* gene expression occurs under conditions of N_2 fixation by all three nitrogenases (Hamilton et al., 2011b).

The *nafY* gene, encoding a FeMo-co insertase, is located downstream of the *rnf* operon but is transcribed from an independent promoter (Rubio et al., 2002). Expression of *nafY* is not absolutely dependent on NifA (hence the *naf* denomination), and *nafY* mRNA accumulates to substantial levels even when growing on NH_4^+ as nitrogen source (Poza-Carrión et al., 2014).

Genes in the *nifB fdxN nifOQ rhdN grx5*^{nif} operon are key to FeMo-co biosynthesis: *nifB* encodes a SAM-radical protein essential to assemble the metal core of FeMo-co, and *nifQ* encodes a protein required for Mo incorporation into the cofactor. Expression from the *nifB* promoter is activated by NifA, and also by the transcriptional activators of the alternative nitrogenases (Drummond et al., 1996). Expression levels within this operon have been investigated

in detail (Rodriguez-Quinones et al., 1993; Hamilton et al., 2011b). Although all these genes are co-transcribed, a sharp drop in expression was observed between the *nifO* and *nifQ* genes. Moreover, their expression pattern was different depending on metal availability in the medium (Mo, V, or only Fe), suggesting the existence of unknown elements that would regulate expression in this operon.

9.4 THE NifA-NifL-GlnK SYSTEM: A DYNAMIC SIGNAL INTEGRATING SYSTEM THAT MODULATES *nif* GENE EXPRESSION

The NifA-NifL-GlnK complex is key in regulating *nif* gene expression. In *A. vinelandii* this system integrates different environmental and intracellular signals including cell energy levels (ATP/ADP ratio), carbon/nitrogen balance, and redox state, to determine if diazotrophic growth is feasible. Different diazotrophic proteobacteria, such as *A. vinelandii*, *Klebsiella pneumonia*e, and *Pseudomonas stutzeri*, display significant differences in their signal integration mechanisms (Merrick and Edwards, 1995; Desnoues et al., 2003; Dixon and Kahn, 2004; Martinez-Argudo et al., 2005; See also Chapters 10, 34).

The biochemical basis of the NifA-NifL-GlnK complex relies on a series of sensing motifs that have been studied in detail (Little et al., 2002, 2012; Martinez-Argudo et al., 2004b) (Fig. 9.2).

NifL is an evolutionary relative of histidine protein kinases that acts as an anti-activator of NifA. The N-terminal domain of NifL is specialized in sensing intracellular redox potential. It contains two PAS motifs (Per-ARNT-Sim) (Hill et al., 1996; Macheroux et al., 1998), one of them carrying a flavin adenine dinucleotide (FAD) cofactor. These PAS domains undergo conformational changes, depending on the redox state of the cell. NifL presents an ATP-binding domain at its C-terminus that belongs to the GHKL (Gly-His-Lys-Leu) superfamily of ATPases (Blanco et al., 1993). This ATP-binding domain seems to perform two different functions: (i) it senses the energy status of the cell by binding ADP and (ii) it senses cellular nitrogen/carbon ratios through an interaction with GlnK, a PII-like protein (Little et al., 2000, Rudnick et al., 2002). Finally, a highly conserved histidine residue is located in the central region of NifL (Histidine-domain) where it seems to play an important role in the transmission of PAS and GHKL signals modulating the interaction of NifL with NifA (Little et al., 2007).

NifA is an EBP that activates transcription of σ^{54}-dependent promoters (Studholme and Dixon, 2003) (Fig. 9.2). The N-terminal part of NifA presents a GAF domain (cGMPphosphodiesterase adenylate cyclase FhlA domain) that binds 2-oxoglutarate (2-OG) for NifA allosteric control (Little and Dixon, 2003; Martinez-Argudo et al., 2004a). 2-OG is a Krebs cycle intermediate that provides not just a direct measure of cellular carbon status but also an indirect measure of cellular nitrogen status. This is because 2-OG is a substrate of the glutamine oxoglutarate aminotransferase (GOGAT) enzyme, being a main source of carbon skeletons for amino acid biosynthesis (Helling, 1998). In addition, NifA presents a central catalytic AAA^+ domain that can interact with the σ^{54} RNA polymerase subunit (Neuwald et al., 1999), and a C-terminal helix-turn-helix DNA-binding domain that recognizes NifA-UAS located 5′ of *nif* genes.

When *A. vinelandii* cells are growing under ideal diazotrophic conditions – nitrogen starvation and high respiratory rates – the level of oxygen is low and the levels of 2-OG and ATP are high. Allosteric binding of 2-OG to the GAF domain produces a conformational change in NifA that impairs NifL binding. Free NifA is able to activate *nif* gene expression through DNA binding and activation of the σ^{54} factor. On the other hand, at relatively low 2-OG levels or, most importantly, when NifL FAD group is oxidized by excess oxygen, NifL is competent enough to inhibit NifA activity (Fig. 9.3). However, additional fine-tuning applies, and is described below (Martinez-Argudo et al., 2005).

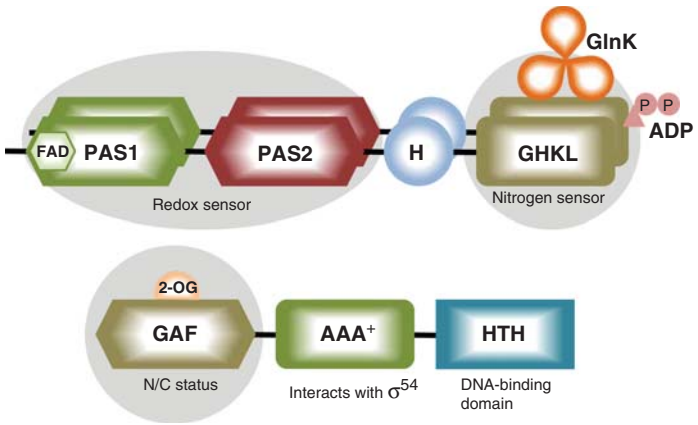

Figure 9.2 Schematic domain architecture of *A. vinelandii* NifL and NifA proteins.

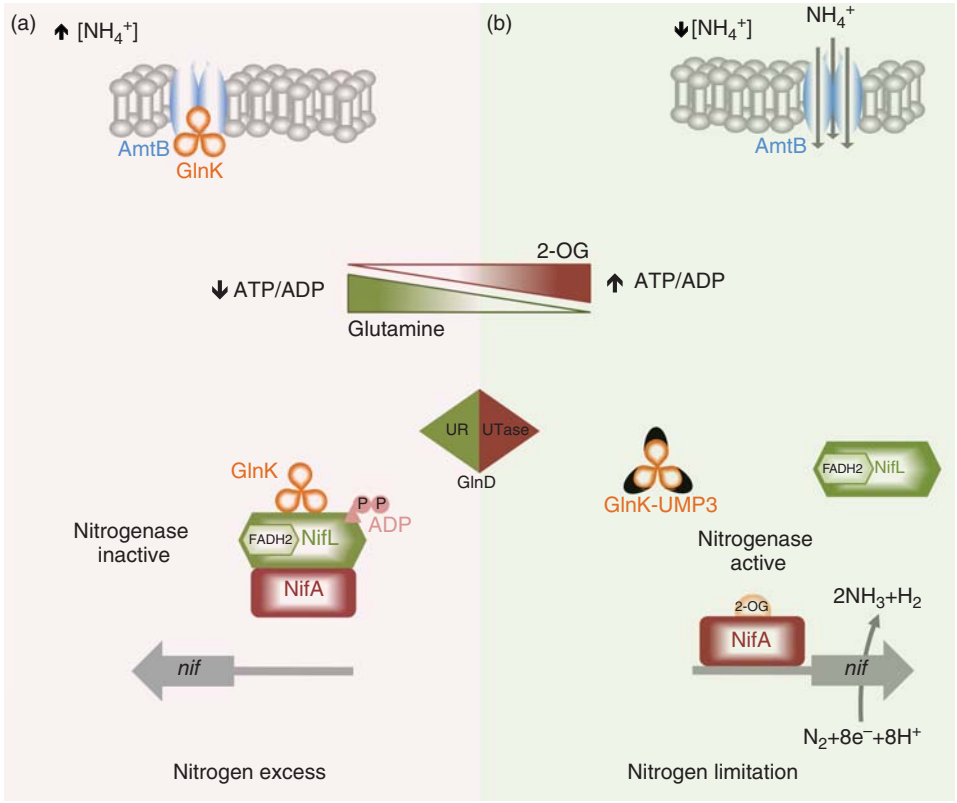

Figure 9.3 GlnK-NifL-NifA response to environmental and metabolic conditions in *A. vinelandii*. (a) Conditions of nitrogen excess result in high concentration of glutamine that leads to deuridylylation of GlnK by the uridylyl-removing (UR) activity of GlnD. The unmodified form of GlnK can interact with (1) AmtB to block active transport of ammonium and (2) with NifL in a GlnK-NifL-NifA ternary complex to block activation of *nif* gene transcription. (b) Conditions of nitrogen limitation result in high concentration of 2-OG that lead to uridylylation of GlnK (GlnK-UMP3) by the uridylyltransferase (UTase) activity of GlnD. The modified form of GlnK is unable to interact with either AmtB or NifL. In addition, high 2-OG levels induce a conformational change in NifA that prevents NifL inhibition and allows binding to specific UAS activating *nif* gene transcription.

A. vinelandii relies on GlnK (a member of the PII family of proteins) and GlnD (an uridylyltransferase/uridylyl-removing enzyme) to determine its cellular nitrogen/carbon balance (Meletzus et al., 1998). The *glnK* gene is co-transcribed with *amtB* in the *glnK amtB* operon and, contrary to the well-studied case of enteric bacteria, including the diazotrophic enterobacterium *K. pneumoniae*, its expression appears to be independent of NtrC (Meletzus et al., 1998). Regulation of *nif* expression in *A. vinelandii* diverges from other diazotrophs in that NtrC is not required for expression (Toukdarian and Kennedy, 1986). This suggests the existence of other proteins that may modulate *nif* transcription. In this context, new transcriptional regulatory regions in the *nifL* codifying region have been described (Mitra et al., 2005).

Uridylylation of GlnK by GlnD plays a key role in *nif* gene activation of expression (Little et al., 2002). GlnD mutants exhibited an Nif^- phenotype (Colnaghi et al., 2001; Rudnick et al., 2002). However, it is possible to revert this phenotype by certain suppressor mutations affecting glutamine synthetase activity (Rudnick et al., 2002).

Under conditions of nitrogen excess (high nitrogen/carbon ratio), non-uridylylated GlnK can interact with the C-terminal domain of NifL, increasing its inhibitory effect over NifA and forming a ternary complex (Fig. 9.3). *In vivo* and *in vitro* experiments have demonstrated this interaction (Little et al., 2002; Rudnick et al., 2002). Unmodified GlnK is also capable of interacting with the cytoplasmic ammonium transporter AmtB (Coutts et al., 2002).

On the other hand, under conditions of nitrogen limitation (low nitrogen/carbon balance) GlnD uridylylates GlnK ($GlnK\text{-}UMP_3$), which cannot interact with NifL. In addition, 2-OG binding to NifA prevents its inhibition by NifL. Interestingly, there is no evidence of NifL interaction with cytoplasmic membranes as it was shown in *K. pneumoniae* where NifL could be immobilized in the membrane, and therefore, hijacked from NifA, resulting in activation of *nif* transcription (Klopprogge et al., 2002).

9.5 PERSPECTIVES

Recently, whole Transcriptome Shotgun Sequencing (RNA seq) conducted on *A. vinelandii* growing diazotrophically with either Mo or V or Fe-only revealed the extent of cross-talk between the three different nitrogenase systems and their integration with central metabolic processes (Hamilton et al., 2011b). This analysis was performed comparing steady-state mRNA levels of NH_4^+-grown cells versus N_2-growth cells. How these mRNA levels are established and how they change over time has been shown recently (Poza-Carrión et al., 2014). This study of *nif* gene expression kinetics provides a detailed picture that reveals new regulatory elements. In this context, we have

observed that a *fdxN* mutant maintains very high levels of *nifA* expression (but not *nifL*) compared to the wild type. Likewise, sharp differences in mRNA levels from genes expressed from a common promoter could be explained by the existence of non-coding RNA elements, but this regulatory aspect has not yet been investigated.

When considering the transfer of *nif* genes to a new organism, a decision must be made on whether to completely imitate the regulatory scheme of a model diazotroph or to wipe out all intrinsic regulatory sequences and use a synthetic biology approach instead (Temme et al., 2012). The more we learn about *nif* regulation the more we will be able to tune our choices (see Chapter 108).

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Chapter 10

Regulatory Coupling of Nitrogen and Carbon Metabolism in Nitrogen-Fixing *Pseudomonas stutzeri* A1501

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10.1 INTRODUCTION

The ability to use nitrogen gas as the sole nitrogen source confers to nitrogen-fixing bacteria an ecological and adaptive advantage for proliferating in natural ecosystems that in general are nitrogen-limiting environments. But, it also imposes considerable physiological constraints, due to the high-energy requirement for nitrogenase functioning, to the extreme oxygen sensitivity of the nitrogenase enzyme and to the complex regulatory mechanisms that prevent expression of the nitrogen-fixation (*nif*) genes under conditions noncompatible with the nitrogen-fixation process (Rees and Howard, 2000; Lawson and Smith, 2002; Dixon and Kahn, 2004). Indeed, diazotrophs are in most cases able to fix nitrogen only under nitrogen-limiting conditions in the presence of a suitable source of carbon and energy and in an environment where their nitrogenase enzyme can be protected from oxygen damage. Therefore, nitrogen-fixing bacteria have evolved several mechanisms to sense multiple environmental signals in order to adapt the nitrogen-fixation process to their physiological constraints (Dixon and Kahn, 2004; see also Chapter 9).

Pseudomonas stutzeri strain A1501, isolated from the rice rhizosphere in China, has emerged as an interesting model system of root-associated nitrogen-fixing bacteria (Qiu et al., 1981; Desnoues et al., 2003; Yan et al., 2013). This strain is one of the rare examples of nitrogen-fixation capacity within the *Pseudomonas* genus *sensu stricto* (Vermeiren et al., 1999; Lalucat et al., 2006; Yan et al., 2013).

P. stutzeri A1501 can survive in the soil, colonize the root surface, and invade endophytically the root tissues of the host plant (You et al., 1995; Rediers et al., 2003; Yan et al., 2013). Therefore, the potential of the strain to adapt to the nutritional environment of the rhizosphere is of particular importance. The nucleotide sequence of the complete genome of *P. stutzeri* A1501 has been determined (Yan et al., 2008). The genome of another nitrogen-fixing *P. stutzeri* strain DSM4166 isolated from the rhizosphere of a "high-fixing" *Sorghum nutans* cultivar in Germany (Krotzky and Werner, 1987) has also been determined (Yu et al., 2011). Interestingly, the nitrogen-fixation genes are carried on a 49-kb genomic island almost identical in both strains (Yan et al., 2013; see also Chapter 21).

The genes of the global regulatory networks controlling nitrogen and carbon sources utilization in *P. stutzeri* A1501 are located in the core genome (Fig. 10.1a). During evolution, A1501 acquired a nitrogen-fixation island with a *nif*-specific regulatory system from a diazotrophic common ancestor (Yan et al. 2008). Consequently, the expression of *nif* genes in *P. stutzeri* is controlled by two regulatory

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systems of different evolutionary origins (Yan et al., 2010, 2013). In this review, we report recent advances made toward understanding the regulatory networks coupling carbon and nitrogen metabolism, including the discovery of small regulatory noncoding RNAs (ncRNAs), and their role in the control of nitrogen fixation of *P. stutzeri* A1501.

10.2 GLOBAL REGULATORY NETWORKS CONTROLLING NITROGEN FIXATION AND NITROGEN SOURCE UTILIZATION IN DIAZOTROPHIC PROTEOBACTERIA

Availability of a nitrogen source is a key regulatory signal controlling the nitrogen-fixation process through complex regulatory networks. Global regulation involves a number of signal transduction and effector proteins such as the *nif*-specific regulators, NifLA, and the global nitrogen regulators, AmtB–GlnK–NtrBC (Javelle et al., 2003; Merrick, 2004).

In several nitrogen-fixing Gammaproteobacteria (e.g., *Azotobacter vinelandii*, *P. stutzeri*, *Klebsiella pneumoniae*), the NifA activator and the antiactivator NifL proteins, encoded by the *nifLA* operon, control the expression of all other *nif* genes. This operon is in turn controlled by the general nitrogen regulatory protein NtrC and by the PII protein (GlnB or GlnK) in response to the fixed nitrogen status (Dixon, 1998; Martinez-Argudo et al., 2004; Xie et al., 2006; see Chapter 9).

One of the best-studied regulatory cascades is that established in *A. vinelandii* (Little et al., 2001, 2002). In this bacterium, 2-oxoglutarate is the metabolic signal controlling the interactions of GlnK with its target genes and acting as a regulator of the interaction between NifL and NifA. Under nitrogen-limiting conditions, GlnK is uridylylated and does not interact with NifLA, and NifA is activated by binding 2-oxoglutarate and can activate transcription of the *nif* operons. Under nitrogen excess GlnK binds to NifL and NifA, forming a complex that cannot activate the transcription of the *nif* operons (see Chapter 9).

10.3 THE NITROGEN REGULATORY CASCADE IN *P. stutzeri* A1501

The *P. stutzeri* A1501 strain is able to grow on ammonium ions or on various nitrogen sources and fix nitrogen under microaerobic and nitrogen-poor conditions (Desnoues et al., 2003). The nitrogen regulatory cascade in A1501 comprises the AmtB–GlnK–NtrBC global nitrogen regulation proteins, which sense the nitrogen signal and subsequently control expression of the *nif*-specific regulatory proteins NifLA (Xie et al., 2006; He et al., 2008; Zhang et al., 2012). These genes have been mapped on the chromosome of *P. stutzeri* A1501 and DSM4166 (Fig. 10.1a,b).

A1501 contains two ammonium transporter genes, *amtB1* and *amtB2*, linked to the *glnK* gene encoding a protein from the PII family (Fig. 10.1a). A *glnKamtB* gene cluster is commonly found in bacteria; but the tandem repeat of ammonium transporter genes found in *P. stutzeri* has not been found in other *Pseudomonas* species nor in the closely related *Azotobacter* (Fig. 10.1c,d) and may be unique to *P. stutzeri* (Vermeiren et al., 2002; He et al., 2008).

Construction of a double *amtB1–amtB2* deletion did not result in a mutant strain showing a growth defect, under the conditions tested, when compared to the growth of the wild-type strain (Zhang et al., 2012). Addition of ammonium

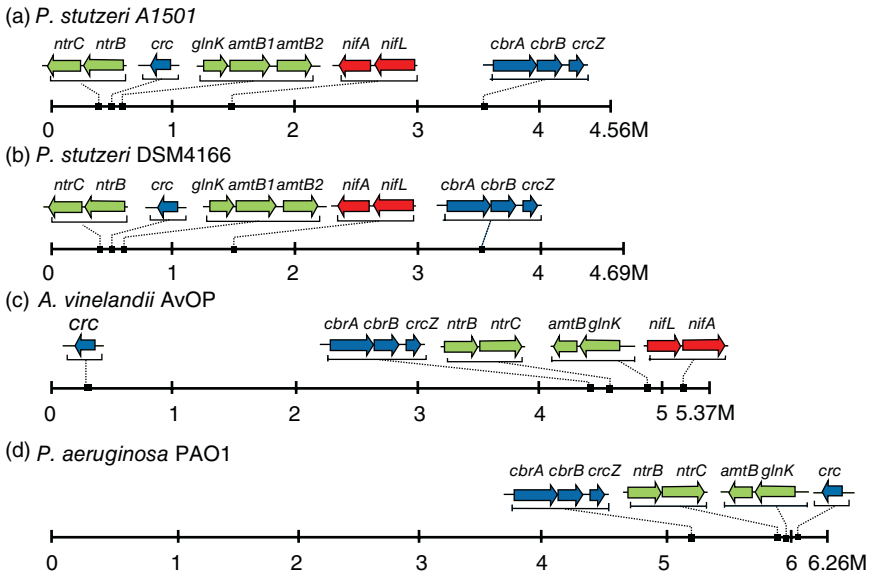

Figure 10.1 Localization of the gene clusters encoding regulatory proteins for nitrogen fixation, global nitrogen regulation, and carbon catabolite control on a linear map of the chromosome of three diazotrophic strains *P. stutzeri* A1501 (a), *P. stutzeri* DSM4166 (b), *A. vinelandii* AvOP (c), and one nondiazotrophic *P. aeruginosa* PAO1 (d).

to a nitrogen-fixing culture had a very small influence on nitrogenase activity in the *amtB* double deletion strain. In contrast, the wild-type strain showed significantly reduced nitrogenase activity, suggesting that the two AmtB proteins are involved in regulating the expression of the nitrogenase genes or nitrogenase activity in response to ammonium (Zhang et al., 2012).

Different strategies have been reported to obtain ammonium excretion by mutant strains (Christiansen-Weniger and Van Veen, 1991). For example, a *nifL* mutant of *A. vinelandii* has been found to excrete ammonium (Bali et al., 1992; Brewin et al., 1999). In the case of A1501, ammonium is excreted in the medium by the *amtB1–amtB2* double mutant strain under nitrogen-fixation conditions, and the amount of ammonium is increased when the strain expresses *nifA* constitutively (Zhang et al., 2012).

PII signal-transduction proteins are known to play a major role in the overall regulation of nitrogen metabolism, including nitrogen fixation (Merrick, 2004; Pedrosa and Elmerich, 2007). In *K. pneumoniae*, two paralogous gene copies *glnB* and *glnK* have been identified, whereas A1501 and *A. vinelandii* encode only a single PII protein termed GlnK (Ninfa and Atkinson, 2000; Rudnick et al., 2002; He et al., 2008). In the case of A1501, transcription of *glnK* is decreased in the presence of ammonium and is partly dependent on NtrC and RpoN under nitrogen-limiting conditions (He et al., 2008). Inactivation of *glnK* leads to a mutant strain devoid of nitrogenase activity and auxotrophic for glutamine (He et al., 2008). Using a *glnK–lacZ* fusion it has been shown that under nitrogen-limiting conditions, *glnK* is expressed from an RpoN-dependent promoter requiring NtrC as a transcriptional enhancer. An interaction between NifL and GlnK was observed in *P. stutzeri* (He et al., 2008), as also reported in *A. vinelandii* (Rudnick et al., 2002; see Chapter 9).

In Proteobacteria, such as *A. vinelandii* and *P. stutzeri*, GlnK is subject to reversible covalent modification by UTase/UR (uridylyltransferase/uridylyl-removing enzyme) encoded by the *glnD* gene (Arcondéguy et al., 2001). We observed that a mutation in the *glnD* gene of *P. stutzeri* prevented the activation of *nif* genes transcription, which resulted in a Nif$^-$ phenotype (unpublished data). It was hypothesized that the nonuridylylated form of GlnK interacts with NifL to inhibit NifA activity. This interaction was prevented when GlnK was fully uridylylated under nitrogen-fixation condition, as shown in *A. vinelandii* (Arcondéguy et al., 2001; see Chapter 9). A schematic representation of the transcriptional regulation of *nif* gene expression in A1501, mediated by the *nif*-specific and the Ntr regulatory systems, is shown in Figure 10.2.

It is particularly interesting to note that many genes of unknown function may play some essential role in controlling the expression of the *nif* genes or the activity of nitrogenase. Indeed, a transcriptome analysis revealed that the expression of a number of genes was significantly altered in response to ammonia sufficient or ammonia limiting conditions or after ammonia shock (Yan et al., 2010, 2013). Among differently regulated genes, nine transcriptional

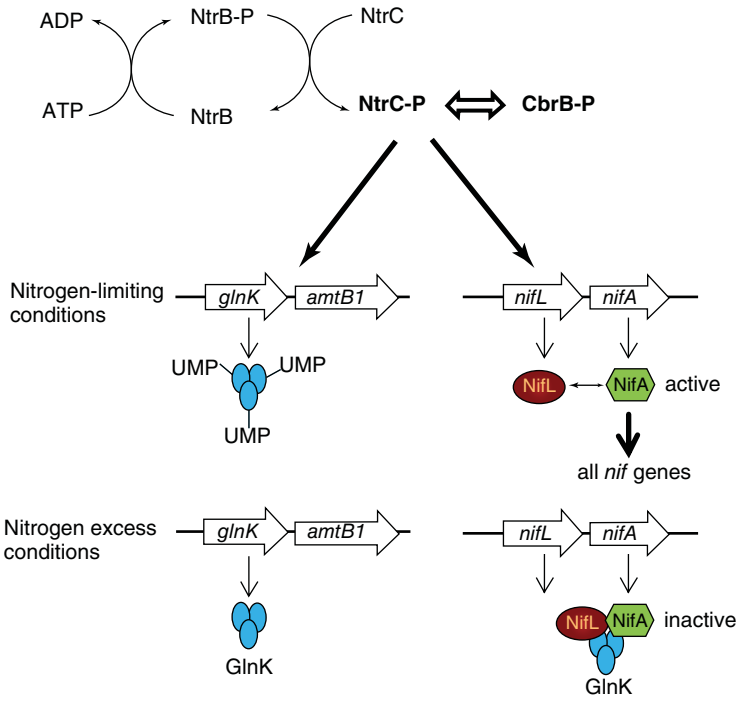

Figure 10.2 Cascade regulation of *nif* genes in *P. stutzeri* A1501 in response to the fixed nitrogen status. Under nitrogen-limiting conditions, glnK is uridylylated and NtrB phosphorylates NtrC, leading to activation of transcription of the *glnK*, *amtB*, and *nifLA* operons. Expression of GlnK prevents NifL from inhibiting NifA, leading to activation of *nif* transcription. Double solid arrow indicates that NtrC and CbrB may have partially overlapping functions. (*Source*: This figure was drawn according to the *Azotobacter* model proposed by Dixon and Kahn (2004) and adapted to data obtained in *P. stutzeri* A1501.)

regulators or outer membrane proteins were identified. The construction of mutant strains was performed, and it was shown that some showed a significant decrease in nitrogenase activity (Yan et al., 2010). However, the possible role of these genes in electron transfer, redox and nonredox catalysis, and sensing of regulatory processes requires further investigation.

10.4 CATABOLITE REPRESSION OF CARBON SOURCES UTILIZATION IN *Pseudomonas*

The rhizosphere is the primary site of interaction between microorganisms and host plants (Starkey, 1958). Bacterial growth in the rhizosphere depends on various carbon sources derived from root exudates (Starkey, 1958). In many cases, bacteria can selectively use a preferred carbon source by preventing the use of secondary substrates. This regulatory process is called catabolite repression control (CRC) and can be achieved by different regulatory mechanisms (Gorke and Stulke, 2008; Rojo, 2010). A classical example is the diauxic growth of *Escherichia coli* in media containing both glucose and lactose, discovered by Jacques Monod in 1941 (MacGregor et al., 1992; Gorke and Stulke, 2008). Catabolite repression is likely to play a role in natural habitats, where different bacterial species are in competition for available nutrients, by optimizing their metabolic versatility (Silby et al., 2011).

CcpA is the main regulator of catabolite repression found in *Bacillus subtilis* (Gorke and Stulke, 2008; Moreno et al., 2001). The Crc protein is the global transcription regulator involved in CRC in *Pseudomonas* (Gorke and Stulke, 2008; Rojo, 2010). Crc acts as a translational repressor by binding to target mRNAs. Crc activity is modulated by the products of the *cbrAB–crcZ* operon that has been particularly well studied in *Pseudomonas aeruginosa* (Sonnleitner and Haas, 2011). Changes in carbon and nitrogen concentration are sensed by the two-component system CbrA/CbrB, which controls expression of the small ncRNA CrcZ (407 nt). This small RNA by binding to the Crc protein can remove Crc from its target mRNA in the absence of the preferred carbon sources and relieve catabolite repression (Sonnleitner et al., 2009). Apart from catabolite repression, it was found that Crc also controls a number of other function, such as twitching motility, biofilm formation, virulence, and quorum sensing (Linares et al., 2010; O'Toole et al., 2000; Parvatiyar et al., 2005).

The two-component CbrA/CbrB system was initially shown to control the utilization of several amino acids and other nitrogenous compounds as sole source of carbon and nitrogen in *P. aeruginosa* (Nishijyo et al., 2001). Therefore, the involvement of *crc*, *CbrAB–crcZ* in carbon catabolite repression and in regulation of nitrogen fixation was investigated. These four genes are present in *Azotobacter* and *P. stutzeri* and have been mapped on their genomes (Fig. 10.1a–c).

10.5 CATABOLITE REPRESSION CONTROL: PROPERTIES OF A *CRC* MUTANT STRAIN OF *P. stutzeri* A1501

Like other metabolically versatile *Pseudomonas* species, *P. stutzeri* A1501 can assimilate a wide range of carbon compounds and live under a wide range of environmental conditions (Silby et al., 2011). Little is known of the role of Crc in the regulation of nitrogen fixation, despite the important role of carbon supply to this process. Especially the connections to nitrogen and carbon metabolism remain to be explored. Our recent studies aimed at assessing the regulatory role of Crc in nitrogen-fixing *P. stutzeri*, emphasizing the potential molecular mechanisms underlying cross talk between carbon and nitrogen metabolism.

P. stutzeri A1501, as many other *Pseudomonas* species, can utilize a variety of aromatic compounds. Our recent study of the β-ketoadipate pathway suggested that benzoate utilization was subject to catabolite repression (Li et al., 2010). Based on nucleotide sequence comparison, we identified a putative *crc* gene in the A1501 genome (Li et al., 2010; see Fig. 10.1a), whose translation product shared the highest similarity (88% amino acid identity) with the well-studied *P. aeruginosa* PAO1 Crc protein. To understand the role of the Crc-like protein of A1501 in catabolite repression, a mutant strain was constructed (Yan et al., 2012a). Subsequently, the *ben*, *cat*, *pca*, and *pob* genes, encoding key enzymes of the aromatic catabolic pathways, were selected for real-time polymerase chain reaction (RT-PCR) analysis. Expression of these selected genes was clearly higher in the *crc* mutant strain than in the parental strain, confirming our earlier observation that benzoate degradation in A1501 was subject to carbon catabolite repression. We further showed that in a medium containing both glucose and lactate, lactate was used more efficiently than glucose by the wild-type strain, indicating that lactate was the preferred carbon source for A1501 growth; whereas the *crc* mutant used glucose as efficiently as lactate.

Interestingly, the *crc* mutant was also impaired in its motility properties and in its competitive root colonization ability (manuscript in preparation). These additional phenotypes were in agreement with former reports on a *P. aeruginosa crc* mutant defective in type IV pilus-mediated twitching motility (Linares et al., 2010). Moreover, inactivation of *crc* resulted in reduced expression of the *glnK*, *ntrC*, *nifA*, and *nifH* genes, in agreement with the reduced nitrogenase activity (30%) compared with that of the wild-type A1501 strain (manuscript in preparation).

10.6 CATABOLITE REPRESSION CONTROL: TRANSCRIPTOME ANALYSIS

Transcriptome analysis and complementary phenotypic and physiologic assays have been performed in different bacteria (*B. subtilis*, *E. coli*) to identify the set of genes controlled by catabolite repression (Moreno et al., 2001; Yoshida et al., 2001; Liu et al., 2005). In the case of *Pseudomonas*, increasing evidence suggest that Crc affects not only specific catabolic pathways but also many other genes required for the interaction of the bacterium with the environment. A mutation in the *crc* gene of *Pseudomonas* was found to have pleiotropic effects, including quorum sensing, and susceptibility to antibiotics and virulence, all of which are important for the establishment of a successful association between the bacteria and their hosts (O'Toole et al., 2000; Parvatiyar et al., 2005; Gorke and Stulke, 2008; Linares et al., 2010).

In order to understand the role of Crc in the regulation of *nif* genes, we have compared the genome-wide transcriptional profile of A1501 wild type to that of a *crc* mutant under nitrogen-fixation conditions. A total of 498 genes were up- or downregulated in the *crc* mutant under nitrogen-fixation conditions. Among those, almost all the *nif* genes and general nitrogen regulatory genes were significantly downregulated. However, their mRNA did not reveal a putative Crc-binding site, suggesting that Crc impaired the expression of the *nif* genes through an unidentified indirect mechanism. Furthermore, a significant proportion (70%) of the 498 genes encoded proteins were involved in aromatic compound catabolism, energy generation, and amino acid biosynthesis and metabolism, and also in the group of stress response-related proteins such as a cold-shock protein (CspD), an osmotically inducible protein (OsmC), a superoxide dismutase (SodC), and two catalases (KatE and KatB), which may collectively contribute to the capacity to protect nitrogenase enzymes under microaerobic nitrogen-fixation conditions (Yan et al., 2012a). Taken together, these data revealed that Crc is a global regulator within the regulatory network coupling between carbon and nitrogen metabolism, which facilitates nitrogen fixation and environmental adaptation of root-associated *P. stutzeri* A1501. This study has extended our knowledge of the Crc regulon in pseudomonads (Fig. 10.3).

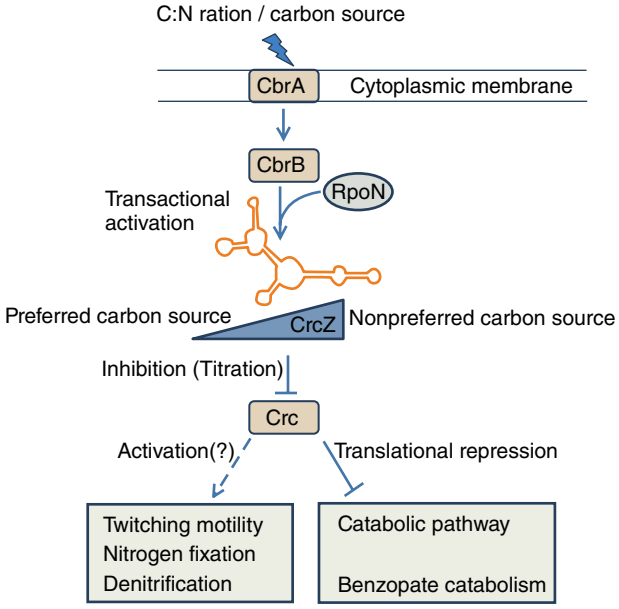

Figure 10.3 A model for the involvement of the CbrA/CbrB/CrcZ/Crc regulatory cascade in the regulation of twitching motility, nitrogen fixation, denitrification, and benzoate catabolism in *P. stutzeri* A1501. In response to a signal sensed by the CbrAB two-component system, the response–regulator CbrB is phosphorylated and activates the expression of CrcZ. CrcZ which harbors several Crc binding sites counteracts Crc function by sequestration of the protein. The question mark means that gene expression activation by Crc is likely to be indirect. (*Source*: This figure was drawn according to Sonnleitner and Haas (2011) and adapted to data obtained in *P. stutzeri* A1501.)

10.7 CATABOLITE REPRESSION: THE CbrA–CbrB SYSTEM IN *P. stutzeri* A1501

Available evidence suggests that in certain species of *Pseudomonas*, the CbrA/CbrB system functions as a global regulatory system required for the assimilation of carbon and nitrogen sources (Nishijyo et al., 2001; Li and Lu, 2007; Zhang and Rainey, 2008). The CbrA/CbrB system forms networks with other regulation systems, such as CrcZ and Crc, which play an important role in the process of microbial adaptation to the environment (Rojo, 2010). In *Pseudomonas putida*, lack of CbrB affects not only carbon metabolism and amino acid utilization but also apparently unrelated functions, such as tolerance to metals, motility, chemotaxis, and the transition from a planktonic lifestyle to the formation of surface-attached biofilm communities (Amador et al., 2010).

Multiple sequence alignment revealed that the CbrAB ortholog in *P. stutzeri* A1501 shared 78% and 84% amino acid identity with the well-characterized *P. aeruginosa* CbrA and CbrB proteins, respectively (unpublished data). A *cbrAB* double mutant strain of A1501 was constructed; its growth properties in LB or lactate containing minimal media did not differ from the wild type. An assay for 95 carbon or nitrogen substrates, using commercially Biolog plates, revealed that the *cbrAB* mutant strain had a growth defect on 20 carbon sources, including seven amino acids, two carbohydrates (α-D-glucose and maltose) and putrescine. In addition, the

cbrAB mutant displayed reduced nitrogenase activity and reduced motility phenotype (manuscript in preparation). However, the mechanism by which these regulators controls nitrogen fixation remain to be elucidated.

10.8 CbrAB AND NtrBC OVERLAPPING FUNCTIONS IN *P. stutzeri* A1501

Sequence comparison revealed that CbrAB belong to the NtrBC family. Although the signal sensed by the CbrAB system remains unknown, this system has been suggested to act coordinately with NtrBC to maintain the carbon–nitrogen balance (Nishijyo et al., 2001). In *Pseudomonas fluorescens*, the CbrAB system regulates operons for the utilization of amino acids that can be used as carbon and nitrogen sources, which are also regulated by the NtrBC system (Zhang and Rainey, 2008). Using catabolism of arginine and histidine as two models, further evidence supports the notion that the CbrAB and NtrBC two-component systems work coordinately to control the carbon and nitrogen metabolic flows in *P. aeruginosa* (Li and Lu, 2007).

To further document the role of CbrB and NtrC in *P. stutzeri* A1501, we compared properties of single *ntrC* and *cbrB* mutants and of an *ntrC/cbrB* double mutant (manuscript in preparation). Specific nitrogenase activity of the three mutant strains was significantly reduced compared to that of A1501, while the specific nitrogenase activity of the double mutant was much lower than that of two single mutant strains, suggesting that CbrB and NtrC may at least partially substitute for each other in the control of nitrogen fixation, as pointed out previously for *P. aeruginosa* in which NtrC and CbrB may have partially overlapping functions (Li and Lu, 2007). To further confirm this possibility, we constructed an *ntrC* mutant strain overexpressing the *cbrB* gene. As expected, overexpression of *cbrB* partially restored nitrogenase activity of the *ntrC* mutant strain. Given that CbrB may at least partially compensate for the regulatory defect caused by the *ntrC* mutation at some operons related with nitrogen fixation, it seemed attractive to speculate that the two-component system represents a link between carbon and nitrogen metabolisms (Fig. 10.4).

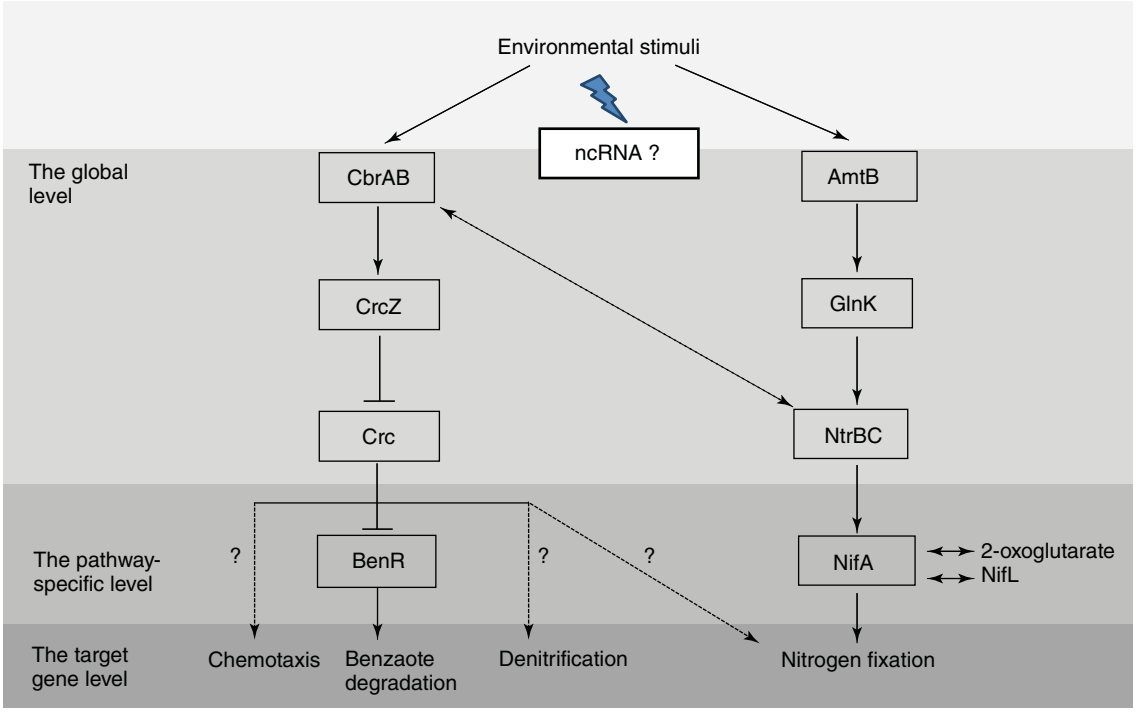

Figure 10.4 Regulatory networks operating in *P. stutzeri* A1501. There are two regulatory cascades in which three levels can be distinguished: (i) the global level: the PII protein, the two-component regulatory proteins and small regulatory ncRNAs, (ii) the specific-pathway level: the key regulators, and (iii) the target gene level: the genes involved in nitrogen fixation, denitrification, benzoate degradation, chemotaxis, and so on. Solid arrows indicate activation of gene expression. Repression is noted as "⊥." Double arrows show the interaction of NifL with NifA or binding of 2-oxoglutarate to NifA. Question marks represent the regulatory steps of unknown mechanism. Dotted double arrow represents a possible link between the two regulatory cascades. For details and references, see text.

Table 10.1 Experimentally found noncoding RNAs in *Pseudomonas*

NcRNA	Size (nt)	Process Regulated	Strain/References
PrrF1	~110; 146	Iron homeostasis	*P. aeruginosa* (Wilderman et al., 2004); *P. syringae* (Filiatrault et al., 2010)
PrrF2	~110; 146	Iron homeostasis	*P. aeruginosa* (Wilderman et al., 2004); *P. syringae* (Filiatrault et al., 2010)
PhrS	212	Quorum sensing	*P. aeruginosa* (Sonnleitner and Haas 2011)
PhrD	72	—	*P. aeruginosa* (Sonnleitner et al., 2008)
PrrH	~325	Iron and heme homeostasis	*P. aeruginosa* (Oglesby-Sherrouse and Vasil, 2010)
PrrB	132	Secondary metabolites production	*P. fluorescens* (Aarons et al. 2000)
RsmX	119; 119	Quorum sensing, swarming motility, and antibiotic compound production	*P. fluorescens* (Kay et al., 2005); *P. brassicacearum* (Lalaouna et al., 2012)
RsmY	124; 118; 118; 121	Biofilm formation	*P. aeruginosa* (Lapouge et al., 2008); *P. fluorescens* (Kay et al., 2005); *P. brassicacearum* (Lalaouna et al., 2012); *P. syringae* (Filiatrault et al., 2010)
RsmZ	~120; 127; 127; 166; 160	Biofilm formation	*P. aeruginosa* (Lapouge et al., 2008); *P. fluorescens* (Kay et al., 2005); *P. brassicacearum* (Lalaouna et al., 2012); *P. stutzeri* A1501 [this work]; *P. syringae* (Filiatrault et al., 2010)
CrcZ	407; 368; 339	Catabolite repression	*P. aeruginosa* (Sonnleitner et al., 2009); *P. putida* (Moreno et al., 2012); *P. stutzeri* A1501 [this work]
CrcY	368; 315	Catabolite repression	*P. putida* (Moreno et al., 2012); *P. stutzeri* A1501 [this work]
RgsA	120	Oxidative stress response	*P. aeruginosa* (Gonzalez et al., 2008)
P1	300; 179	—	*P. aeruginosa* (Livny et al., 2006); *P. syringae* (Filiatrault et al., 2010)
tmRNA	353; 386	—	*P. aeruginosa* (Sonnleitner et al., 2008); *P. syringae* (Filiatrault et al., 2010)
P5	90	—	*P. aeruginosa* (Gonzalez et al., 2008)
P8	130	—	*P. aeruginosa* (Livny et al., 2006)
P9	128	—	*P. aeruginosa* (Livny et al., 2006)
P11	100	—	*P. aeruginosa* (Livny et al., 2006)
P15	180	—	*P. aeruginosa* (Livny et al., 2006)
sRNA1714	200	—	*P. aeruginosa* (Gonzalez et al., 2008)
P18	100	—	*P. aeruginosa* (Livny et al., 2006)
P24	300; 241	—	*P. aeruginosa* (Livny et al., 2006); *P. syringae* (Filiatrault et al., 2010)
sRNA2315	180	—	*P. aeruginosa* (Gonzalez et al., 2008)
P26	250; 65	—	*P. aeruginosa* (Livny et al., 2006); *P. syringae* (Filiatrault et al., 2010)
RNase P	350; 292; 365	Precursor tRNA splicing	*P. aeruginosa* (Gonzalez et al., 2008); *P .syringae* (Filiatrault et al., 2010); *P. stutzeri* A1501 [this work]
P35	62	—	*P. aeruginosa* (Livny et al., 2006)
sRNA2626	200	—	*P. aeruginosa* (Gonzalez et al., 2008)
P32	80	—	*P. aeruginosa* (Livny et al., 2006)
P34	150	—	*P. aeruginosa* (Livny et al., 2006)
PhrX	151	—	*P. aeruginosa* (Sonnleitner et al., 2008)
PhrY	195	—	*P. aeruginosa* (Sonnleitner et al., 2008)

(*continued*)

Table 10.1 (*Continued*)

NcRNA	Size (nt)	Process Regulated	Strain/References
6S RNA	183; 178; 178	Transcriptional regulation of many promoters	*P. aeruginosa* (Sonnleitner et al., 2008); *P.syringae* (Filiatrault et al., 2010); *P. stutzeri* A1501 [this work]
yybP-ykoY	141	—	*P. syringae* (Filiatrault et al., 2010)
t44	152	—	*P. syringae* (Filiatrault et al., 2010)
FMN	166	—	*P. syringae* (Filiatrault et al., 2010)
Cobalamin	224; 214; 198	—	*P. syringae* (Filiatrault et al., 2010)
SRP_bact	99; 190	Ribonucleoprotein implicated in the translation and targeting of proteins to cell membranes	*P. syringae* (Filiatrault et al., 2010); *P. stutzeri* A1501 [this work]
P16	195	—	*P. syringae* (Filiatrault et al., 2010)
S15	111	—	*P. syringae* (Filiatrault et al., 2010)
TPP	104	—	*P. syringae* (Filiatrault et al., 2010)

10.9 SMALL REGULATORY NONCODING RNAs

Research on the discovery and characterization of small regulatory ncRNAs; also named sRNAs) in bacteria has exploded in recent years (Waters and Storz, 2009). In particular, a number of ncRNA molecules were identified in *Pseudomonas* species (Table 10.1). Active research is focused on the mechanisms by which these ncRNAs adjust bacterial physiology in response to environmental clues (Sonnleitner et al., 2012).

To date, very few small regulatory ncRNAs have been functionally characterized in nitrogen-fixing bacteria. Reports include an internal antisense RNA that regulates expression of the photosynthesis gene *isiA* in the cyanobacterium *Synechocystis* sp. PCC 6803 (Dühring et al., 2006), a ncRNA required for growth of freshwater cyanobacterium *Synechococcus elongatus* strains PCC 6301 under multiple stress conditions (Nakamura et al., 2007), and four *Rhizobium etli* ncRNAs varying during free-living growth and during interaction with the eukaryotic host plant (Vercruysse et al., 2010).

10.10 IDENTIFICATION OF ncRNA IN THE GENOME OF *P. stutzeri* A1501

The RNA transcripts synthesized by A1501 under nitrogen fixation and ammonium repression conditions were sequenced using Illumina (Solexa)'s high-throughput sequencing platform. A total of 53 ncRNAs induced under nitrogen-fixation conditions were identified. Among these candidates, 17 ncRNA-encoding genes had higher transcriptional levels (>2 fold) while 6 genes were down regulated (<0.5-fold) under nitrogen-fixation conditions (Yan et al., 2012b). The BLASTN searches of the 53 ncRNA sequences against GenBank database showed that (i) 12 ncRNAs were specific to A1501 because no homologous sequences were found in any other bacteria; (ii) 24 ncRNAs were only found in some *P. stutzeri* strains; and (iii) 17 ncRNAs were found to be conserved in other species of *Pseudomonas* or in other bacteria.

The BLASTN of Rfam database indicated that seven genes were highly conserved among bacteria and encoded previously known functional classes of ncRNAs (Table 10.1). In *P. aeruginosa*, the GacS/GacA two-component system positively controls the transcription of sRNA RsmZ, which is crucial for the expression of genes involved in virulence (Kay et al., 2005). The 6S RNA is a small ncRNA that interacts with σ^{70}-RNA polymerase and downregulates transcription at many promoters during the stationary phase. It has been proposed that 6S RNA mimics the conformation of DNA during transcription initiation, suggesting that the contacts between RNA polymerase and 6S RNA or DNA may be similar (Klocko and Wassarman, 2009). P15 is a ncRNA that was predicted using bioinformatic tools in the genome of the opportunistic pathogen *P. aeruginosa* and its expression verified by Northern blot analysis, but its function is unknown (Livny et al., 2006). The 407 nt ncRNA CrcZ comprises five CA-motifs and has been shown to antagonize the function of Crc in catabolite repression. In *P. aeruginosa*, the transcription of *crcZ* is driven by CbrAB in an RpoN-dependent manner (Sonnleitner et al., 2009). A conserved RpoN-binding site (GG-N10-GC) located in the promoter region of the A1501 *crcZ* gene was identified. Under nitrogen-fixation conditions, the expression of *crcZ* was almost completely repressed in the *rpoN* mutant of A1501 (Yan et al., 2012b; manuscript in preparation).

We employed the computational software sRNATarget to predict their potential targets. Some nitrogen-fixation-

related genes within the *nif*-island were predicted to be potential targets of 18 ncRNAs (13 upregulated ncRNAs and five down-regulated ncRNAs), suggesting that these ncRNAs are probably involved in the regulation of nitrogen fixation in *P. stutzeri* A1501. Further work is necessary to identify target genes of these ncRNAs and to determine as to whether they are involved in the regulation of nitrogen fixation in *P. stutzeri* and other diazotrophs.

10.11 CONCLUDING REMARKS

Diazotrophs have evolved transcriptional and posttranslational mechanisms to control the nitrogen-fixation process depending on environmental conditions. The mechanisms operating in *P. stutzeri* are extremely complex. They include the following: (i) a classical nitrogen regulatory cascade found in *Gammaproteobacteria*, involving two main systems, namely the *nif*-specific regulators, NifLA, and the global nitrogen regulators, AmtB–GlnK–NtrBC. The cascade found in *P. stutzeri* A1501 is close to that of *Azotobacter* (see Chapter 9). (ii) The CbrAB–CrcZ–Crc system for carbon catabolite repression. Data reported here strongly suggest that the catabolite repression cascade regulates also nitrogen fixation in *P. stutzeri* A1501. The involvement of this cascade in *Azotobacter* that is carrying the same genes is unknown. (iii) A complex network of ncRNAs. As the synthesis of several of them was specifically upregulated under nitrogen-fixation conditions, it is believed that some are playing a role in integrating nitrogen fixation with global cellular physiology. (iv) An unidentified mechanism (revealed by the analysis of the *amtB* mutant strains), that to some extent, controls nitrogenase activity and synthesis.

The complexity of the nitrogen-fixation regulatory network is schematized in Figure 10.4, with an emphasis on a possible cross talk between various regulatory systems as well on fine-tuning of nitrogen fixation with overall cellular physiology (Yan et al., 2012c).

Hopefully, the understanding of these complex regulations, especially during association with the host plant, should contribute to a rational exploitation of such fine-tuned interactions and provide various accessible alternative strategies for the genetically engineering of diazotrophic strains with higher potential in their transfer of fixed nitrogen to the host plant.

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Chapter 11

Regulation of Nitrogen Fixation and Molybdenum Transport in *Rhodobacter capsulatus*

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11.1 INTRODUCTION

Rhodobacter capsulatus is a phototrophic Alphaproteobacterium capable of growing with atmospheric dinitrogen (N_2) as sole nitrogen source (Weaver et al., 1975). Nitrogen fixation, the reduction of N_2 to ammonia (NH_3), is catalyzed by two genetically distinct nitrogenases: a molybdenum-dependent nitrogenase (Mo-nitrogenase) and an alternative Mo-free iron-only nitrogenase (Fe-nitrogenase) (Schneider et al., 1991; Schüddekopf et al., 1993; see Chapter 2).

R. capsulatus carries more than 50 genes involved in nitrogen fixation, the majority of which is clustered in one of four chromosomal regions (Masepohl and Klipp, 1996; Strnad et al., 2010). Among these are the "classical" nitrogen fixation (*nif*) genes including the structural genes of Mo-nitrogenase, *nifHDK*, and genes involved in iron-molybdenum cofactor (FeMo-co) biosynthesis, *nifB*, *nifUSVW*, and *nifENX*. Apart from these *nif* genes, *R. capsulatus* contains alternative nitrogen fixation (*anf*) genes including the structural genes of Fe-nitrogenase, *anfHDGK* (Schüddekopf et al., 1993). The *rnf* (*Rhodobacter* nitrogen fixation) genes are essential for diazotrophic (N_2-dependent) growth using either Mo- or Fe-nitrogenase (Schmehl et al., 1993; Schüddekopf et al., 1993). The *rnf* genes, which were first described for *R. capsulatus*, but later on also identified in many other bacteria, encode a membrane complex involved in electron supply of both nitrogenases (Jouanneau et al., 1998; Kumagai et al., 1997; Saeki and Kumagai, 1998).

Mo-nitrogenase exhibits higher specific N_2-reducing activity than Fe-nitrogenase and thus appears to be the preferred enzyme providing sufficient molybdate supply (Schneider et al., 1997). In *R. capsulatus*, Mo-nitrogenase activity depends on two transporters with different molybdate affinities. At nanomolar molybdate concentrations in the environment, the high-affinity ATP-binding cassette (ABC) transporter ModABC is essential for Mo-nitrogenase activity (Wang et al., 1993). At micromolar molybdate concentrations, Mo-nitrogenase activity depends on the oxyanion permease PerO, which belongs to the ArsB/NhaD family (Gisin et al., 2010). To date, PerO is the only experimentally confirmed bacterial molybdate transporter outside the ABC transporter family.

This review summarizes our current knowledge on regulation of nitrogen fixation and molybdenum transport in *R. capsulatus*. Currently, more than 20 proteins are known or predicted to control expression and activity of Mo-nitrogenase, Fe-nitrogenase, and molybdate uptake (Table 11.1). This review focuses on nitrogen and molybdenum regulations, while the regulatory effects of oxygen and light will only briefly be touched upon.

11.2 TRANSCRIPTIONAL ACTIVATION OF NITROGEN FIXATION GENES

The *R. capsulatus* nitrogen regulation (*ntr*) genes *ntrB* and *ntrC* encode a cognate two-component system acting on top

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Table 11.1 Genes involved in the regulation of nitrogen fixation

Gene	Accession Number	Known or Predicted Function(s) of Gene Product	References
nifA1	rcc03267	Transcriptional activator of Mo-nitrogenase (*nif*) genes	Masepohl et al. (1988)
nifA2	rcc00567	Transcriptional activator of Mo-nitrogenase (*nif*) genes	Masepohl et al. (1988)
anfA	rcc00584	Transcriptional activator of Fe-nitrogenase (*anf*) genes	Schüddekopf et al. (1993)
rpoN	rcc00568	Nitrogen regulation-specific sigma factor (alias NtrA, $Sigma^{54}$, or NifR4)	Kranz and Haselkorn (1988)
ntrC	rcc01798	TCS response regulator (alias NifR1)	Kranz and Haselkorn (1988)
ntrB	rcc01797	TCS sensor kinase (alias NifR2); phosphodonor toward NtrC	Kranz and Haselkorn (1988)
ntrY	rcc01799	TCS sensor kinase; phosphodonor toward NtrC	Drepper et al. (2006)
regA	rcc00045	TCS response regulator; transcriptional activator of *nifA2*	Elsen et al. (2000)
mopA	rcc00561	Molybdate-responsive repressor of *anfA* transcription	Kutsche et al. (1996)
mopB	rcc00560	Molybdate-responsive repressor of *anfA* transcription	Kutsche et al. (1996)
ranR	rcc02599	Putative TetR-like regulator required for Fe-nitrogenase-dependent growth	Sicking et al. (2005)
ihfA	rcc01912	IHF α-subunit involved in DNA bending of RpoN-dependent promoters (alias HimA)	Toussaint et al. (1991)
ihfB	rcc01126	IHF β-subunit involved in DNA bending of RpoN-dependent promoters (alias Hip)	Toussaint et al. (1993)
nrfA	rcc01801	Hfq-like protein enhancing *nifA1*, *nifA2*, and *anfA* expression	Drepper et al. (2002)
hvrA	rcc00046	Histone-like protein modulating *nif* gene expression	Raabe et al. (2002)
glnB	rcc01673	PII-like signal transduction protein interacting with NtrB, NifA1, NifA2, and DraT	Pawlowski et al. (2003)
glnK	rcc03387	PII-like signal transduction protein interacting with NifA1, NifA2, and DraT	Pawlowski et al. (2003)
amtB	rcc03386	Ammonium transporter required for PII sequestration to the membrane	Tremblay et al. (2007)
glnD	rcc00466	Uridylyltransferase/UMP-removing enzyme acting on PII proteins	Annotation by sequence similarity
draT	rcc03016	Dinitrogenase reductase ADP-ribosyltransferase acting on NifH and AnfH	Masepohl et al. (1993)
draG	rcc03017	Dinitrogenase reductase activating glycohydrolase	Masepohl et al. (1993)

TCS, two-component regulatory system; IHF, integration host factor.

of a regulatory cascade controlling expression of Mo- and Fe-nitrogenase (Fig. 11.1) (Cullen et al., 1996; Masepohl and Klipp, 1996). Under nitrogen-fixing conditions, the response regulator NtrC becomes phosphorylated, and thus, activated (Cullen et al., 1996). In the following step, NtrC∼P activates transcription of its target genes including *nifA1*, *nifA2*, and *anfA* (Table 11.2) (Bowman and Kranz, 1998). Importantly, *anfA* transcription is strongly repressed by molybdate, thus limiting Fe-nitrogenase expression to conditions unfavorable for Mo-nitrogenase. *R. capsulatus* NtrC activates target promoters recognized by RNA polymerase containing the housekeeping sigma factor RpoD, while NtrC proteins from other diazotrophs typically activate promoters depending on the alternative sigma factor RpoN (alias NtrA) (Foster-Hartnett et al., 1994; see Chapter 10).

NtrC is absolutely essential for diazotrophic growth of *R. capsulatus* using either the Mo- or the Fe-nitrogenase (Masepohl et al., 2001; Schüddekopf et al., 1993). The sensor kinase NtrB serves as a phosphodonor for NtrC as demonstrated by *in vitro* reconstitution of the two-component system (Cullen et al., 1996). In addition to NtrB, the sensor kinase NtrY is an efficient *in vivo* phosphodonor as implied by gene disruption studies (Fig. 11.1) (Drepper et al., 2006). In contrast to single mutants lacking either NtrB or NtrY, an *ntrB–ntrY* double mutant is no longer able to fix dinitrogen, suggesting that NtrB and NtrY are the only physiological phosphodonors for NtrC. In line with this assumption, acetyl phosphate does not phosphorylate *R. capsulatus* NtrC *in vitro*, while it is a substrate for enteric NtrC (Cullen et al., 1996).

The *R. capsulatus ntrYX* genes, which are located directly downstream of the *nifR3–ntrBC* genes, encode a two-component system involved in oxygen regulation of photosynthesis genes (Gregor et al., 2007). Transcription of the *ntrYX* operon is primarily driven by the *ntrY* promoter but, in addition, requires the *nifR3* promoter for maximum expression (Drepper et al., 2006). A similar order of *nifR3–ntrBC* and *ntrYX* genes is conserved in different bacteria. Recently, *Brucella abortus* NtrY was shown to contain a redox-sensing heme cofactor, which controls autokinase activity (Del Carmen Carrica et al., 2012). Only at low ambient oxygen concentrations, the heme iron is in its reduced

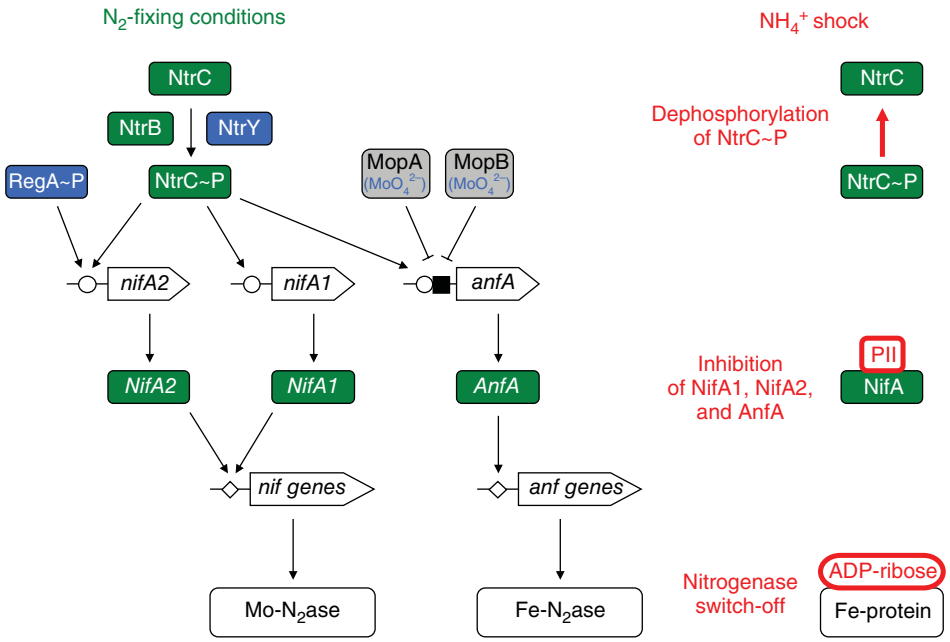

Figure 11.1 **Regulatory cascades controlling nitrogen fixation**. Proteins involved in nitrogen, oxygen, and molybdenum regulation of nitrogen fixation are shown in green, blue, and gray, respectively. Phosphorylation of the response regulator NtrC depends on two sensor kinases, NtrB and NtrY. NtrC~P activates RpoD-dependent promoters (open circles), while NifA1, NifA2, and AnfA activate RpoN-dependent promoters (open diamonds). MopA and MopB repress *anfA* by binding the *anfA* Mo-box (black square). Upon ammonium addition to an N_2-fixing culture, NtrC~P is dephosphorylated (and thus, inactivated) and, upon interaction with either of two PII proteins, GlnB and GlnK, NifA1 and NifA2 are inhibited. Nitrogenase switch-off involves ADP-ribosylation of the Fe-protein and an ADP-ribosylation-independent mechanism (not depicted in this figure). For further details, see text.

Table 11.2 Genes directly activated by NtrC

Target Genes	Accession Numbers	Known or Predicted Functions	References
nifA1	rcc03267	Activator of *nif* gene transcription	Masepohl et al. (1988)
nifA2	rcc00567	Activator of *nif* gene transcription	Masepohl et al. (1988)
anfA	rcc00584	Activator of *anf* gene transcription	Schüddekopf et al. (1993)
mopA–modABCD	rcc00561–rcc00565	Molybdate-responsive regulator and molybdate transporter	Wang et al. (1993)
glnB–glnA	rcc01673–rcc01674	PII-type signal transduction protein and glutamine synthetase	Foster-Hartnett and Kranz (1994)
glnK–amtB	rcc03387–rcc03386	PII-type signal transduction protein and ammonium transporter	Drepper et al. (2003)
amtY	rcc01792	Ammonium transporter	Yakunin and Hallenbeck (2002)
ureDABCEFG	rcc01217–rcc01224	Urease	Masepohl et al. (2001)

form, which is a prerequisite for autophosphorylation of NtrY and subsequent phosphotransfer to NtrX. It is tempting to speculate that in *R. capsulatus*, the phosphorylation states of NtrB and NtrY reflect the fixed nitrogen and ambient oxygen concentrations, respectively, and cross talk to NtrC integrates both signals (Fig. 11.1).

In addition to NtrC, the response regulator RegA acts as a coactivator of *nifA2* but not *nifA1* (Fig. 11.1) (Elsen et al., 2000). RegA and its cognate sensor kinase RegB comprise a global redox-responding two-component system involved in regulation of many important processes including photosynthesis, carbon fixation, and (via *nifA2*) nitrogen fixation (for a recent review, see Wu and Bauer, 2008). At low ambient oxygen concentrations, RegA becomes phosphorylated and, in turn, activates its target promoters. Apparently, *nifA2* regulation by NtrC and RegA provides another mechanism integrating nitrogen and oxygen signals. Furthermore, RegA directly interacts with the above-mentioned NtrX protein (Gregor et al., 2007). RegA–NtrX interaction likely reduces the amount of homomeric RegA protein and, hence, affects *nifA2* control in response to oxygen tension.

NifA1, NifA2, and AnfA activate transcription of all the other *nif* and *anf* genes required for expression and activity of the Mo- and Fe-nitrogenases. In contrast to

NtrC, which activates RpoD-dependent promoters, NifA1, NifA2, and AnfA activate RpoN-dependent promoters as is the case for their homologs in other diazotrophs (Hübner et al., 1993; Schüddekopf et al., 1993). The *rpoN* gene forms part of the RpoN-dependent *nifU2–rpoN* operon, and therefore, RpoN appears to be autoactivated under nitrogen-fixing conditions (Cullen et al., 1994; Preker et al., 1992). In addition to the RpoN-dependent promoter preceding the *nifU2–rpoN* operon, a weak constitutive promoter mediates basal expression of *rpoN* required to initiate the autoregulatory circuit.

11.3 AMMONIUM INHIBITION OF NITROGEN FIXATION

In ammonium-grown *R. capsulatus* cells, *nifR3-ntrBC* expression is comparably high as in nitrogen-fixing cells (Cullen et al., 1998). Furthermore, NtrC levels remain constant during growth with ammonium and dinitrogen, but transcriptional activation of *nifA1*, *nifA2*, and *anfA* occurs exclusively under nitrogen-fixing conditions. Altogether, these findings suggest that, in the presence of ammonium, NtrC predominantly occurs in its unphosphorylated (inactive) form.

Addition of ammonium to a nitrogen-fixing culture causes at least three effects as depicted in the regulatory model in Figure 11.1 (Drepper et al., 2003). First, an ammonium shock leads to dephosphorylation of NtrC∼P and, hence, inactivation of the regulator stopping further transcription of *nifA1*, *nifA2*, and *anfA*. Second, the transcriptional activators NifA1, NifA2, and AnfA are posttranslationally inhibited, thus preventing further transcription of Mo- and Fe-nitrogenase genes. Third, Mo- and Fe-nitrogenase activities are specifically inhibited by ammonium (Jouanneau et al., 1983; Masepohl et al., 1993). In addition, removal of light leading to energy deprivation also inhibits Mo-nitrogenase activity (Pierrard et al., 1993; Yakunin and Hallenbeck, 2002). In contrast to the well-studied nitrogen-signaling pathway, the energy-signaling pathway remains largely unknown.

Signal transduction after an ammonium shock involves two PII-like proteins, GlnB and GlnK (Fig. 11.2) (Drepper et al., 2003). A *glnB–glnK* double knockout strain is still able to synthesize active Mo- and Fe-nitrogenases demonstrating that none of the PII proteins is *per se* essential for nitrogen fixation. However, proper regulation of nitrogen fixation requires both proteins, which mutually depend on each other (Tremblay et al., 2007).

R. capsulatus GlnB and GlnK specifically interact with different protein partners involved in nitrogen regulation as shown by yeast two-hybrid studies (Pawlowski et al., 2003). Apart from the formation of homo- and heteromers, both PII proteins interact with NifA1, NifA2, and DraT (dinitrogenase reductase ADP-ribosyltransferase), the latter enzyme catalyzing ADP-ribosylation of the nitrogenase Fe-protein (see later). Furthermore, GlnB (but not GlnK) interacts with NtrB. Notably, none of the two signal transduction proteins interacts with AnfA [our unpublished results]. Interaction of the *R. capsulatus* PII proteins with their partners is likely to be modulated by 2-oxoglutarate, the ATP–ADP ratio, and by reversible uridylylation reflecting the cellular carbon, energy, and nitrogen status, as shown for PII proteins of diverse species (Fokina et al., 2010; Gerhardt et al., 2012; Jiang and Ninfa, 2009; Tremblay et al., 2007; Tremblay and Hallenbeck, 2009; Truan et al., 2010; Xu et al., 2001).

Disruption of the *R. capsulatus glnB* gene strongly diminishes ammonium inhibition of *nifA1* and *anfA* transcription, suggesting that GlnB is critical to keep NtrC in its unphosphorylated (inactive) form (Drepper et al., 2003). As mentioned earlier, GlnB specifically interacts with NtrB (Pawlowski et al., 2003). Taken together, it seems likely that dephosphorylation of *R. capsulatus* NtrC∼P involves an NtrB–GlnB complex as in *Escherichia coli* (Jiang et al., 1998; see also Chapter 9).

R. capsulatus synthesizes two highly similar NifA regulators, NifA1 and NifA2, which functionally replace each other in *nif* promoter activation (Masepohl et al., 1988; Paschen et al., 2001). Constitutive (NtrC-independent) expression of *nifA1* leads to accumulation of NifA1 protein in the presence of ammonium as shown by Western analysis. However, constitutive expression of *nifA1* or *nifA2* does not relieve ammonium inhibition of *nif* promoters, suggesting that both regulators are posttranslationally inhibited by ammonium. A *glnB–glnK* double mutant synthesizes huge amounts of Mo-nitrogenase in the presence of ammonium, while no expression is observed in single mutants implying that either GlnB or GlnK is sufficient for NifA1 and NifA2 inhibition (Drepper et al., 2003). As mentioned earlier, both regulators directly interact with GlnB and GlnK (Pawlowski et al., 2003). Altogether, these findings support the conclusion that upon an ammonium shock, GlnB and GlnK bind to NifA1 and NifA2 leading to inhibition of both activators.

Similar experiments with a strain constitutively expressing *anfA* indicate that AnfA is also inhibited upon ammonium addition, as described earlier for NifA1 and NifA2 (Drepper et al., 2003). In a *glnB–glnK* double mutant, however, Fe-nitrogenase expression is still inhibited by ammonium, suggesting that AnfA inhibition follows a mechanism other than that controlling NifA1 and NifA2.

Ammonium addition to a nitrogen-fixing *R. capsulatus* culture inhibits *in vivo* Mo-nitrogenase activity (Förster et al., 1999; Pierrard et al., 1993). Rapid and complete "switch-off" is observed in highly nitrogen-limited cultures, while moderately nitrogen-limited cultures only partly inhibit nitrogenase by the "magnitude response" reflecting the amount of added ammonium (Yakunin and Hallenbeck,

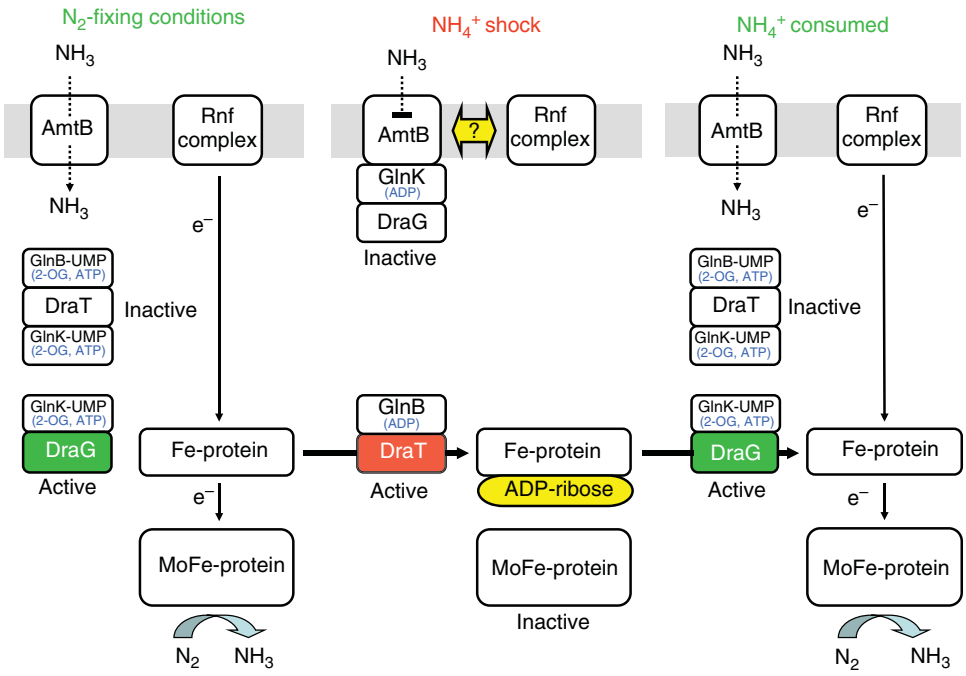

Figure 11.2 **Posttranslational control of nitrogenase activity**. N_2 fixation requires electron flow from the Rnf complex to the Fe-protein and further to the MoFe-protein. Upon an ammonium shock, DraT catalyses ADP-ribosylation of the Fe-protein, which in its modified form is no longer able to deliver electrons to the MoFe protein. When the ammonium is consumed, DraG removes the ADP-ribose moiety from the Fe-protein, thereby restoring its ability to transfer electrons to the MoFe-protein. Activities of DraT and DraG are controlled by various interactions with GlnB and GlnK. AmtB is involved in inactivation of DraG and the Rnf complex by membrane sequestration and yet an unknown mechanism (indicated by question mark), preventing electron transfer to the Fe-protein. For further details, see text.

1998). In *R. capsulatus*, switch-off involves two mechanisms, a slow DraT-dependent and a fast DraT-independent mechanism (Förster et al., 1999; Jouanneau et al., 1983; Pierrard et al., 1993; Tremblay and Hallenbeck, 2009; Yakunin and Hallenbeck, 1998). Similarly, other bacteria including *Azoarcus* and *Azospirillum brasilense* control nitrogenase switch-off by two mechanisms (Oetjen and Reinhold-Hurek, 2009; Zhang et al., 1996). By contrast, DraT-dependent switch-off is the only mechanism regulating nitrogenase activity in *Rhodospirillum rubrum* (Liang et al., 1991; Zhang et al., 1996; see Chapter 12).

DraT and DraG (dinitrogenase reductase activating glycohydrolase) form a two-enzyme system antagonistically, controlling activity of the nitrogenase Fe-protein (for a recent review, see Huergo et al., 2012). Upon ammonium addition, DraT is activated and, in turn, activated DraT modifies the Fe-protein by ADP-ribosylation. Fe-protein modification prevents association with the MoFe-protein and consequently stops electron supply to the MoFe-protein (Fig. 11.2). DraT activation is likely to require complex formation with GlnB-ADP as recently shown for *A. brasilense* DraT-GlnB by *in vitro* studies (Moure et al., 2013; see Chapter 13). Upon ammonium consumption, DraT is inactivated, while DraG becomes active. Activated DraG removes the ADP-ribosyl moiety from the Fe protein, thereby restoring its capacity to donate electrons to the MoFe-protein. DraT and DraG activities are controlled by various interactions with PII proteins, in which complex formation is modulated by small effector molecules and PII uridylylation (Fig. 11.2).

An *R. capsulatus* strain lacking DraT does no longer modify (ADP-ribosylate) NifH and AnfH, the dinitrogenase reductase components of the Mo- and Fe-nitrogenases, respectively, strongly indicating that, in addition to Mo-nitrogenase, the alternative nitrogenase is subject to posttranslational regulation (Masepohl et al., 1993). Mo-nitrogenase switch-off, however, is still observed in the *draT* mutant demonstrating the presence of a second, DraT-independent mechanism (Förster et al., 1999). In line with this finding, strains expressing mutant NifH proteins, which are no longer modified by DraT, still exhibit the switch-off response to ammonium (Pierrard et al., 1993).

The ammonium-induced switch-off is completely abolished in an *R. capsulatus* mutant lacking the ammonium transporter AmtB (Yakunin and Hallenbeck, 2002). AmtB is dispensable for growth with ammonium, but appears to be involved in ammonium retention under nitrogen-fixing conditions. In addition, AmtB might function as an ammonium sensor for the switch-off process. Upon ammonium addition, a ternary AmtB–GlnK–DraG complex is likely to be formed in *R. capsulatus* (Fig. 11.2) as described for *A. brasilense*

AmtB–GlnZ–DraG (Huergo et al., 2007; Rajendran et al., 2011; Tremblay and Hallenbeck, 2008; Tremblay et al., 2007). Assuming that membrane-bound DraG is inactive, DraT-mediated modification of the Fe-protein will not be reversed until DraG is released again.

As mentioned earlier, *amtB* disruption abolishes both the DraT-dependent and the DraT-independent ammonium responses (Yakunin and Hallenbeck, 2002). The DraT-independent switch-off mechanism probably prevents electron flow from the Rnf complex to the Fe-protein (Fig. 11.2) as recently suggested for *Azoarcus* (Sarkar et al., 2012).

11.4 MOLYBDENUM REPRESSION OF Fe-NITROGENASE AND Mo TRANSPORT

Expression of Fe-nitrogenase enables *R. capsulatus* to grow diazotrophically under conditions of Mo-starvation otherwise unfavorable for growth depending on Mo-nitrogenase. *In vivo* Fe-nitrogenase activity is maximal at ambient Mo concentrations below 1 nM, while activity gradually decreases with increasing Mo concentrations (Wang et al., 1993). Fe-nitrogenase activity is half-maximal at Mo concentrations of about 10 nM, and no longer detectable at 100 nM. This decrease results from Mo-dependent *anfA* repression, confining expression of the central transcriptional activator of Fe-nitrogenase genes, AnfA, to Mo-limiting conditions (Fig. 11.1) (Kutsche et al., 1996; Wiethaus et al., 2006).

Transcription of the *anfA* gene is repressed by two regulators, MopA and MopB, which are structurally and functionally similar to *E. coli* ModE (Fig. 11.1) (Hall et al., 1999; Kutsche et al., 1996; Wang et al., 1993; Wiethaus et al., 2009). ModE-like regulators, which consist of a C-terminal molybdate- and an N-terminal DNA-binding domain, act as one-component regulators sensing and responding to the cellular Mo status. Deletion of both, *mopA* and *mopB*, but not single gene mutations, abolishes *anfA* repression indicating that MopA and MopB substitute for each other regarding *anfA* repression (Kutsche et al., 1996; Wiethaus et al., 2006). Binding of molybdate by MopA and MopB increases affinity of the regulators for the *anfA* Mo-box, which overlaps the transcription start site (Kutsche et al., 1996; Müller et al., 2010; Wiethaus et al., 2006). Mo-boxes are conserved palindromic sequences found in various target promoters (Fig. 11.3). Notably, the isolated DNA-binding domain of MopB efficiently binds the *anfA* promoter *in vitro* (Müller et al., 2011). As one would expect for a truncated MopB protein lacking the Mo-sensing domain, binding of the *anfA* promoter is independent of Mo availability.

The *R. capsulatus mopA* gene forms part of the *mopA–modABC* operon, while *mopB* forms a monocistronic operon located upstream of *mopA* (Fig. 11.3) (Wiethaus et al., 2006). The *mopB* gene is constitutively expressed, while the *mopA–modABC* operon is repressed by MopA and MopB, thereby confining expression of MopA and the high-affinity molybdate transporter ModABC to Mo-limiting conditions. As mentioned earlier for the *anfA* Mo-box, MopA and MopB bind the *mopA* Mo-box, which overlaps the *mopA* transcription start site (Kutsche et al., 1996; Wiethaus et al., 2006).

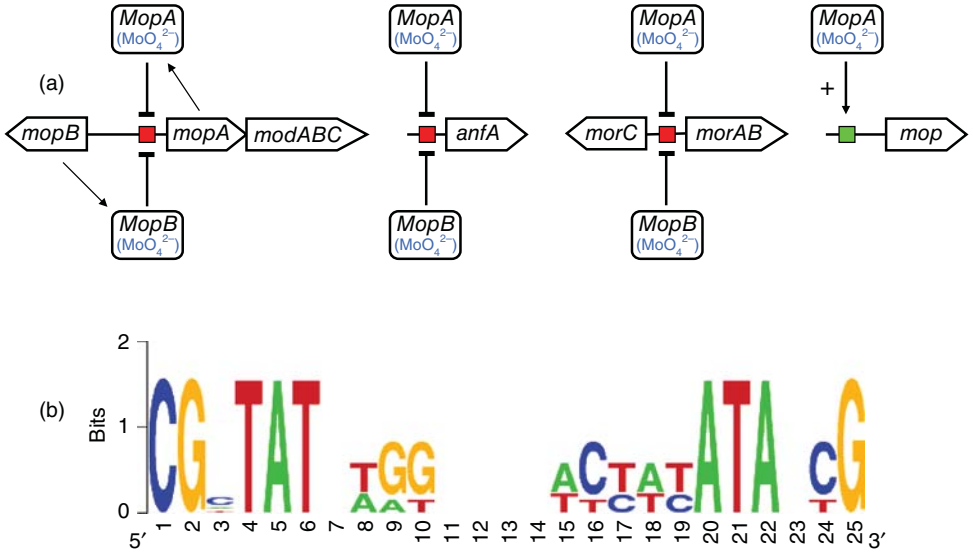

Figure 11.3 Gene regulation by MopA and MopB. (a) The molybdate-responsive one-component regulators MopA and MopB bind the Mo-repressed Mo-boxes upstream of *mopA*, *anfA*, and *morA* (given in red). MopA (but not MopB) binds the Mo-activated *mop* Mo-box (given in green). (b) The Mo-box consensus based on the *mopA*, *anfA*, *morA*, and *mop* Mo-boxes was generated using the weblogo.berkeley.edu program.

Remarkably, *R. capsulatus* wild-type cells exhibit significant Mo-nitrogenase activity even at ambient Mo concentrations as low as 1 nM (Wang et al., 1993). *In vivo* Mo-nitrogenase activity increases with increasing Mo concentrations reaching maximal levels at 100 nM. Mutants lacking ModABC require about 100-fold higher ambient Mo concentrations than the wild type to achieve comparable Mo-nitrogenase activities (Wang et al., 1993; Wiethaus et al., 2006). Double mutants lacking ModABC and the permease PerO require even higher Mo concentrations than *modABC* mutants to express active Mo-nitrogenase (Gisin et al., 2010). These and other findings support the conclusion that ModABC is essential for Mo uptake at nanomolar concentrations in the environment, while PerO is crucial for Mo import at micromolar concentrations. In addition to molybdate, PerO transports other oxyanions including tungstate, vanadate, and sulfate. In contrast to *modABC*, expression of *perO* appears to be independent of Mo availability (Gisin et al., 2010).

R. capsulatus harbors the divergently transcribed Mo-regulated (*mor*) genes, *morAB* and *morC*, coding for a putative ABC transporter exhibiting clear sequence similarity to ModABC (Wiethaus et al., 2006). Both MopA and MopB bind the *morA–morC* intergenic region, which encompasses a highly conserved Mo-box, and repress transcription of *morAB* and, to lesser extent, *morC*. In contrast to ModABC, however, MorABC is dispensable for Mo-nitrogenase activity.

MopA and MopB substitute (at least in part) for each other as repressor proteins controlling *anfA*, *mopA–modABC*, and *morAB* (Fig. 11.3). In addition, MopA acts as an activator of the *mop* gene, which encodes a hexameric molybdate-binding protein possibly involved in Mo-storage (Wiethaus et al., 2006, 2009). Remarkably, MopB does not substitute for MopA in *mop* activation. As one would expect for an activator-binding site, the *mop* Mo-box does not overlap the transcription start site but, instead, is located further upstream. A mutant *mop* promoter, in which the *mop* Mo-box is exchanged against the *anfA* Mo-box, is still activated by MopA demonstrating that an original repressor-binding site may well function as an activator-binding site, provided its correct positioning relative to the other *cis*-regulatory promoter elements (Müller et al., 2010).

The Mop protein specifically interacts with MopB but not with MopA (Wiethaus et al., 2009). It is tempting to speculate that Mop serves as an intracellular Mo buffer specifically donating molybdate to MopB. Alternatively, complex formation may prevent binding of target promoters by the regulator. In any case, Mop–MopB interaction is likely to modulate MopB-dependent gene regulation.

In summary, many details of the complex regulatory network controlling nitrogen metabolism in *R. capsulatus* have already been elucidated, but many open questions remain to be solved. Particularly, the regulatory effects of environmental signals other than nitrogen and molybdenum (e.g., light, oxygen, and iron) require future studies.

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Chapter 12

Metabolic Regulation of Nitrogenase Activity in *Rhodospirillum rubrum*: The Role of PII Proteins and Membrane Sequestration

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12.1 INTRODUCTION

In all diazotrophs, the expression of nitrogenase is regulated in response to the availability of biologically usable nitrogen and to the oxygen concentration in the cellular compartment where nitrogenase is located (Dixon and Kahn, 2004). This regulation can be understood as a means to economize the use of cellular energy, as the reaction catalyzed by nitrogenase has a high ATP demand, nitrogenase is present in high concentrations in the cell and is degraded in the presence of oxygen. Transcriptional regulation does however not lead to a rapid response, as would be required to react to sudden and short-term changes in the environment. In some diazotrophs however, there is yet another level of regulation, primarily sensing changes in the nitrogen supply and in the energy status. The response to an increased availability of nitrogen or a decrease in the energy level is inhibition of nitrogenase activity, a regulation at the metabolic level (Nordlund, 2000).

At the culture level, this control is displayed as the loss of nitrogenase activity when a source of combined nitrogen such as ammonium ions is added to the culture, or when the driving force for cellular ATP production is cut off (Nordlund, 2000). This phenomenon has been termed the "switch-off" effect and was first observed in *Rhodospirillum rubrum*.

In 1949, Kamen and Gest demonstrated that *R. rubrum* could fix nitrogen, and shortly after, they reported that when ammonium ions were added to a culture of nitrogen fixing *R. rubrum* the activity was inhibited, but recovered after a certain time (Gest, 1999; Gest and Kamen, 1949; Kamen, 1986). Schick some 20 years later rediscovered this effect and showed that the duration of inhibition was dependent on the concentration of ammonium ions added (Schick, 1971). Further studies showed that nitrogenase activity was not only inhibited *in vivo*, but the activity in extracts of cells to which NH_4^+ had been added was also lower (Neilson and Nordlund, 1975). Taken together, those reports laid the groundwork for the studies at the molecular level that were to follow.

12.2 THE "SWITCH-OFF" EFFECT AT THE MOLECULAR LEVEL

At the molecular level, the inhibition of nitrogenase activity is due to reversible modification of the Fe-protein of nitrogenase (Nordlund, 2000). In *R. rubrum*, the

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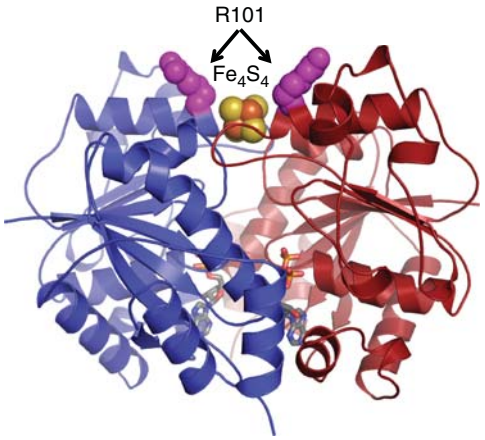

Figure 12.1 Structure of the Fe-protein of nitrogenase. The Fe_4S_4 cluster is indicated as well as the R101 residue (purple) in each subunit, of which only one is modified by ADP-ribosylation. At the bottom of the structure, the binding of one adenine nucleotide to each subunit is shown. This figure was produced using PyMOL. Fe-protein from PDB Id: 1N2C.

modification consists of an ADP-ribose moiety bound to arginine 101 (Pope et al., 1985a, b). The reaction leading to ADP-ribosylation is catalyzed by dinitrogenase reductase ADP-ribose transferase (DraT), whereas the removal of the modification is catalyzed by dinitrogenase reductase activating glycohydrolase (DraG) (Lowery and Ludden, 1988; Lowery et al., 1986; Saari et al., 1984, 1986; see also Chapter 11). With present knowledge about the nitrogenase complex and the docking between its two components, the MoFe-protein (dinitrogenase) and the Fe-protein (dinitrogenase reductase), the inhibitory effect of the modification is easily understood. Arg101 is located close to the Fe_4S_4 cluster donating electrons to the P-cluster in the MoFe-protein, and in the middle of the area involved in the docking to the MoFe-protein (Fig. 12.1). The effect of the ADP-ribosylation is thus that the two proteins cannot interact and no electron transfer can take place.

In Figure 12.2, the "switch-off" effect in *R. rubrum* is schematically depicted. At the addition of a "switch-off" effector, for example, NH_4^+ or glutamine, or the removal of the culture from the light, there is a rapid loss of nitrogenase activity. When the effector added has been metabolized or the light is turned on, nitrogenase activity is regained (Fig. 12.2a). This series of events is due to the modification of the Fe-protein catalyzed by DraT, leading to loss of nitrogenase activity followed by the removal of the ADP-ribose group in the reaction catalyzed by DraG (Fig. 12.2b).

The activity of DraT and DraG are in turn regulated so that they are not active at the same time (Fig. 12.2a). It has been shown that DraT is only active for a short time (Liang et al., 1991), whereas DraG is active except during "switch-off." Both of these regulatory enzymes are active as isolated, which means that the regulation of DraT is lost upon cell breakage.

12.3 THE DraT AND DraG PROTEINS

12.3.1 DraT

DraT is a monomeric protein with a molecular mass of 30 kda (Lowery and Ludden, 1988, 1989; Lowery et al., 1986), catalyzing the modification of the Fe-protein in a reaction where NAD^+ is the donor of the ADP-moiety. MgADP is required for the reaction with the *R. rubrum* Fe-protein as substrate, whereas MgATP is inhibitory. DraT will only ADP-ribosylate the Fe-protein, but not only the one from *R. rubrum*. It has however been shown that some NAD^+ analogs can serve as donor of the ADP-ribose group *in vitro*. When DraT was purified from *R. rubrum* it was noticed that the fraction with activity also contained a contamination running at about 13 kda on SDS-PAGE (Lowery and Ludden, 1988). This can now be explained as we have found that DraT from *R. rubrum* requires GlnB, a PII protein (see below), not only for activity but also for stability (Selao, Teixeira, Nordlund unpublished). In addition, our recent results suggest that binding to GlnB increases the affinity for NAD^+. So far no crystal structure of DraT is available, but it would obviously be important in elucidating its catalytic function and the regulation of its activity possibly by the interaction with GlnB.

To analyze the activity of DraT, the loss of nitrogenase activity can be used, and also more convenient, the fact that only one of the two identical subunits of the Fe-protein is modified (Fig. 12.2). This gives rise to two bands on SDS-PAGE gels using a low crosslinker concentration, with equal amounts of the two bands obtained with fully ADP-ribosylated Fe-protein (Kanemoto and Ludden, 1984).

12.3.2 DraG

R. rubrum DraG has also been purified and characterized. It is a monomeric protein, with a molecular mass of 32 kda (Saari et al., 1984, 1986; Zumft and Nordlund, 1981). In *R. rubrum* extracts, DraG is found associated with the chromatophore membrane; as a first step in the purification, it is solubilized by a washing with 0.5 M NaCl (Ludden and Burris, 1976; Nordlund et al., 1977). The catalysis of the hydrolysis of the ADP-ribose group from the Fe-protein requires the presence of MgATP and another divalent cation. We have shown that Mn^{2+} most likely is the ion functioning *in vivo*, but *in vitro* Fe^{2+} is equally efficient. There is no activity with only Mg^{2+} present *in vitro* (Nordlund and Noren, 1984). In contrast to DraT, DraG will use denatured ADP-ribosylated Fe-protein as substrate as well as some low molecular mass compounds (Pope et al., 1986; Saari et al., 1984, 1986).

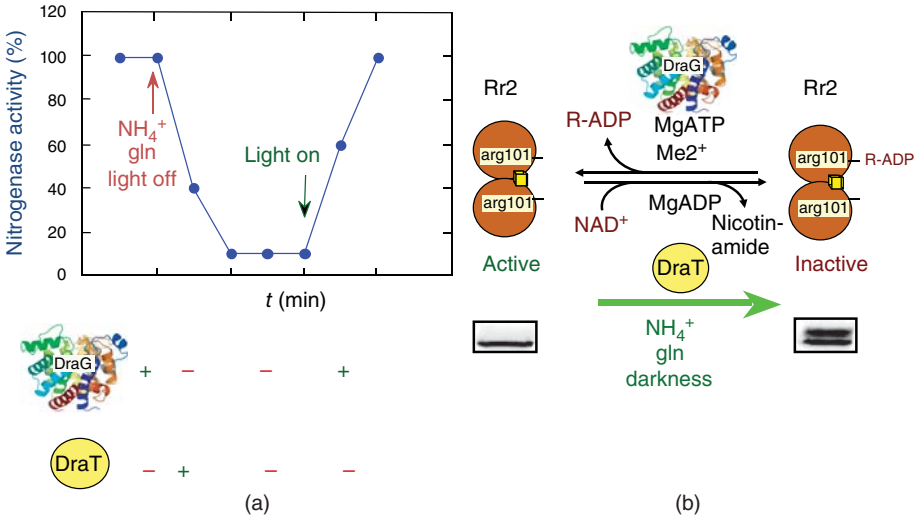

Figure 12.2 Schematic illustration of "switch-off" in *R. rubrum*. (a) The effect on nitrogenase activity upon addition (at red arrow) of ammonium ions (NH_4^+) or glutamine (gln) or turning off the light. Under the diagram, the activity of DraT and DraG before, during, and after the "switch-off" is indicated. (b) Schematic showing the reactions catalyzed by DraT and DraG. Rr2 denotes the Fe-protein from *R. rubrum*. Underneath Rr2, the appearance of the two bands on SDS-PAGE of modified Rr2 is shown.

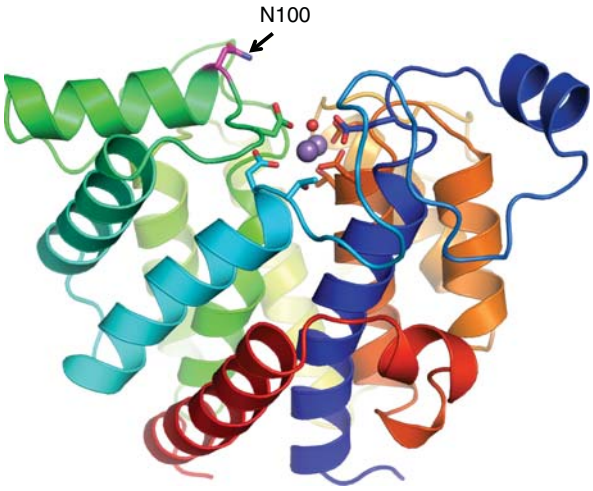

Figure 12.3 The structure of DraG from *R. rubrum*. Asparagine 100 is indicated and the two purple spheres are the two manganese ions in the active site. This figure was produced using PyMOL. DraG from PDB id: 2WOC.

A key to understanding the function of DraG is the 3D structure, which we were recently able to determine for *R. rubrum* DraG (Berthold et al., 2009). The structure (Fig. 12.3) shows an all helical compact protein composed of 16 helices. The active site is located deep in a grove between loops and contains two manganese ions. Based on a structure in which an ADP-ribose was trapped, a catalytic mechanism was proposed (Berthold et al., 2009). This was further verified by specific substitutions of key amino acids (Berthold et al., 2009) and has gained further support from computational studies (Himo, Manta, personal communication).

12.3.3 The *draT* Operon

Both DraT and DraG are constitutively expressed but at very low concentrations in the cell. The *draT* and *draG* genes form an operon located close to the *nifH* gene, encoding the Fe-protein. The *draT* operon is however transcribed in the opposite direction (Fitzmaurice et al., 1989; Liang et al., 1991). In addition to *draT* and *draG*, the *R. rubrum* operon contains a third gene *draB*, located downstream of *draG*, encoding a 16-kda peptide of unknown function (Zhang et al., 2001a).

12.4 REGULATION OF DraT AND DraG ACTIVITIES

One of the key challenges is to understand how DraT and DraG are regulated. Over the years, there have been many proposals, but today it is generally agreed that PII proteins and AmtB are both central to this regulation, at least during nitrogen "switch-off."

12.4.1 PII Proteins and AmtB

PII proteins are homotrimeric proteins of 42 kda that function in signal transduction in regulation and coordination of carbon and nitrogen metabolisms. They have been identified in diazotrophic *Archaea*, all bacterial domains, as well as in the chloroplast of plants and red algae (reviewed in Arcondeguy et al. (2001), Forchammer (2008), and Ninfa and Jiang (2005)). The structure of PII proteins has been solved from a number of organisms and shows a cylindrical core with three protruding loops on the top of the cylinder, the T-loops, one from each subunit. In addition, there are three ATP/ADP binding sites and three binding sites for 2-oxoglutarate. The

binding of these effector molecules induces different conformations of the T-loops. PII proteins interact with other proteins, thereby conveying a signal reflecting the metabolic carbon and nitrogen status as well as the cellular energy level. There are two different means by which PII proteins can be affected by the metabolic status; by binding ATP, ADP, and 2-oxoglutarate; and/or by covalent modification, uridylylation. Clearly, the ATP/ADP ratio reflects the energy status, whereas 2-oxoglutarate is an indicator of the carbon/nitrogen balance. In Protobacteria, the reversible uridylylation is catalyzed by the bifunctional enzyme GlnD and occurs at a conserved tyrosine in the T-loop. GlnD is regulated by the concentration of either glutamine or 2-oxoglutarate, depending on the organism and its metabolism, but in both cases reflecting the nitrogen status (Jiang et al., 1998; Jonsson and Nordlund, 2007). Uridylylation affects the binding of PII proteins to their targets, as this in most cases involves the T-loop.

One PII protein target is the ammonia channel AmtB, a trimeric membrane protein belonging to the Amt/Rh family of transport proteins and mediating transport of ammonia/ammonium ions into the cell (Forchammer, 2008; Khademi et al., 2004; Tremblay and Hallenbeck, 2009). It has been suggested that in addition to the role as a transporter, it is also involved in the regulation of metabolism in response to the extra cellular concentration of ammonium ions (Javelle et al., 2004). It is in this function that PII proteins have a role.

In some seminal work, it was shown that PII proteins form a complex with AmtB in *Escherichia coli*, and that this only occurs with the unmodified form of the PII protein. The structure of the complex has been determined and shows that each T-loop of the PII protein is inserted into one of the three subunits of AmtB, and that the PII protein thereby blocks the transport channels, hindering the influx of ammonia (Conroy et al., 2007; Durand and Merrick, 2006; Javelle and Merrick, 2005).

In most bacteria, there are more than one PII protein. In *E. coli* there are two paralogs, GlnB and GlnK, of which the latter is believed to be the physiologically AmtB-binding PII protein. In *R. rubrum*, there are three paralogs, GlnB, GlnJ, and GlnK (Zhang et al., 2001b). GlnJ is a K-type paralog, and its gene is located in the same operon as the gene encoding AmtB1, an organization that is quite common. There is also an AmtB2 protein in *R. rubrum*, but as for the GlnK protein, no function has yet been identified.

In *R. rubrum*, GlnJ in the unmodified form has been shown to bind to AmtB1, and this occurs under conditions of nitrogen sufficiency (Wang et al., 2005). The role of GlnB is most likely similar to the one in *E. coli*, that is, interacting with other PII protein targets such as GlnE and NtrB.

12.4.2 Regulation of DraT Activity

Upon the addition of "switch-off" effectors, DraT becomes active catalyzing the transfer of the ADP-ribose moiety from NAD^+ to arg101 in one of the subunits of the Fe-protein. However, as isolated DraT is inactive or shows very low activity, the latter may be due to the presence of a (contaminating) PII protein. Relatively little is known of the regulation of DraT, but our current model for nitrogen "switch-off" in *R. rubrum* stipulates that binding of GlnB to DraT leads to activation, presumably by increasing its affinity for NAD^+.

For energy "switch-off," even less is known. It has been shown that when subjecting a culture of *R. rubrum* to darkness, the ratio of NAD^+ to NADH is dramatically increased (Jackson and Crofts, 1968). One possible model would therefore be that the much higher NAD^+ concentration is sufficient to support high DraT activity, even without GlnB binding. It should be noted that we have shown that "switch-off" does not require deuridylylation of neither GlnB nor GlnJ in *R. rubrum* (Teixeira et al., 2010). As uridylylated GlnB/GlnJ has not been shown to be able to bind to AmtB1 or DraT, it can be assumed that GlnB–DraT interaction is not required for DraT activity during energy "switch-off."

12.4.3 Regulation of DraG Activity

It is a consensus today that DraG inactivation is due to sequestration to the chromatophore membrane upon exposure of a *R. rubrum* culture to "switch-off" conditions. Kim et al. (2004) showed that a DraG mutant in which N100 had been substituted for a lysine did not show normal "switch-off," that is, this strain would not show normal decrease in activity. We have made the same substitution and could verify the results by Zhang et al., but could also show that the DraG variant was active *in vitro* and did not bind to the chromatophore membrane. As N100 is located close to the entrance to the catalytic site of DraG, we postulate that upon binding to the membrane, the catalytic site is covered preventing access to the ADP-moiety of the modified Fe-protein. Other substitutions of N100 are also active *in vitro*, but in contrast they are sequestered to the membrane and the "switch-off" effect is normal in these mutants.

A major question is however the identity of the signal(s) that leads to binding to the membrane. A number of candidates have been suggested, but today we see two different processes in *R. rubrum*, one during nitrogen "switch-off" and the other for energy "switch-off." In both cases, we envisage that DraG binds to an unknown membrane protein that changes its conformation from nonbinding to binding of DraG under both conditions. Upon addition of a nitrogen effector, for example, NH_4^+, uridylylated GlnJ becomes deuridylylated in the reaction catalyzed by GlnD and in this form binds to AmtB1. Upon binding of GlnJ, AmtB1

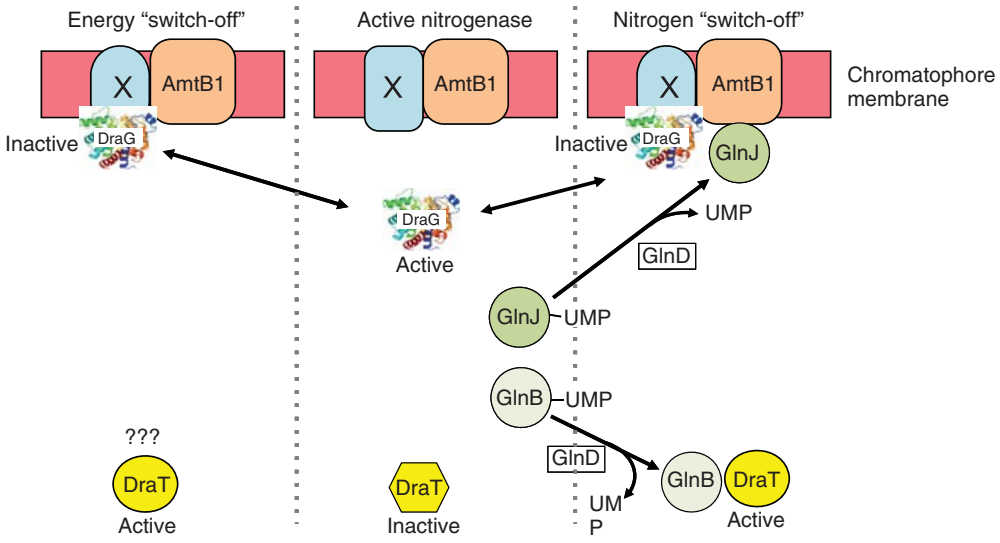

Figure 12.4 Schematic representation of our model of events taking place during "switch-off." In the middle, the situation during high nitrogenase activity is shown, both PII proteins, GlnB and GlnJ, are uridylylated and cannot bind to targets. DraT is inactive and DraG is active. The right part shows the situation during nitrogen "switch-off." GlnJ is bound to AmtB1 after deuridylylation by GlnD. AmtB1 affects the unknown protein (X), making it a binding partner for DraG, which then is inactive. GlnB interacts with DraT after deuridylylation by GlnD, activating DraT. The left part shows the situation during energy "switch-off." Much less is known, but we suggest that X has adopted the DraG-binding conformation because of a lowered membrane potential. The higher DraT activity may be due to an increase in the NAD^+/NADH ratio.

changes its conformation, which in turn causes the unknown DraG-binding protein to adopt a confirmation that promotes binding of DraG. When the NH_4^+ added is metabolized, GlnD catalyzes uridylylation of GlnJ, which then cannot bind to AmtB1. AmtB1 and therefore also the DraG-binding protein, go back to the original conformation. All leading to DraG being released from the membrane.

Much less is known about the process during energy "switch-off," but according to our model turning off the light leads to a change in the membrane potential that can be relayed to the unknown DraG-binding protein, causing a change in its conformation. The model is supported by the fact that even under nitrogen-sufficient conditions, that is, under conditions when neither AmtB1 nor GlnJ are expressed, DraG is found associated with the membrane.

Our model for the regulation of DraT and DraG activities is depicted in Figure 12.4, but it should be emphasized that there may be other factors involved, one such is the redox state of the Fe-protein (Halbleib et al., 2000). It has been shown that the reduced form is a better substrate for DraG, whereas the oxidized Fe-protein is the preferred substrate for DraT. Even if redox is not the major or required signal, it may have some influence during energy "switch-off" as we have shown that the physiological electron transfer leading to reduction of the Fe-protein in *R. rubrum* requires light (Edgren and Nordlund, 2004).

12.5 DraT AND DraG IN OTHER ORGANISMS

The "switch-off" effect has also been studied in other phototrophs, primarily in *Rhodobacter capsulatus* (Tremblay et al., 2007; Tremblay and Hallenbeck, 2008; see Chapter 11), and in *Rhodopseudomonas palustris* (Zumft and Castillo, 1978). In neither of these organisms have, however, detailed studies at the molecular level been reported. This is in contrast to the work on *Azospirillum brasilense*, from which detailed biochemical as well as structural studies have contributed substantially to our understanding of the processes during "switch-off" (Huergo et al., 2007. 2012; Li et al., 2009; Ljungström et al., 1989; Zhang et al., 1997). It should be noted that the model for *A. brasilense* differs in some aspects from the one we propose for *R. rubrum*. One difference is that DraG is inactivated by binding to an unidentified protein in the chromatophore membrane, whereas for *A. brasilense* it is has been suggested that binding to GlnZ leads to inactivation (Rajendranan et al., 2011; see Chapter 13).

As DraT and DraG are expressed under all conditions in *R. rubrum*, it could be suggested that these regulatory proteins have additional functions, perhaps in *R. rubrum* and also in other organisms. A list of some 40 organisms having a *draT* gene was recently published (Huergo et al., 2012). A recent search in the presently sequenced genomes shows that *draT* and *draG* can be found in about 60 genomes. Evidently,

a closer analysis is required to be able to draw conclusions on any possible additional function(s) of these proteins, for example, are they expressed, do they form an operon, and is the organism a diazotroph.

12.6 CONCLUDING REMARKS

Our knowledge about the "switch-off" effect has increased tremendously since the first demonstration by Gest and Kamen about 60 years ago, and even compared to the contribution by Schick 20 years later. Today, the molecular details of the proteins involved are in most cases known, but the interactions between DraT and DraG with their partners in the regulatory mechanism are yet to be clearly established. In spite of great similarities at the protein level, the models for "switch-off" in *R. rubrum* and *A. brasilense* show differences that need to be understood. A major question is the regulatory events at the molecular level during energy "switch-off," be it by darkness in *R. rubrum* or by oxygen removal in *A. brasilense*.

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Chapter 13

How Does the DraG–P_{II} Complex Regulate Nitrogenase Activity in *Azospirillum brasilense*?

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13.1 INTRODUCTION

13.1.1 The *Azospirillum brasilense* Nitrogen-Fixation System

Azospirillum brasilense is an aerobic Alphaproteobacteria diazotroph. This bacterium lives in the soil on its own or is associated with the rhizosphere of various plants fixing nitrogen gas from the atmosphere. Therefore, *Azospirillum* has been utilized as nitrogen biofertilizer and plant growth promoting rhizobacterium (PGPR) by farmers worldwide to improve crop productivity (Bashan et al., 2004). The nitrogen-fixation genes (*nif*) are responsible for the nitrogen-fixation ability; these genes are highly conserved among all nitrogen-fixing bacteria. The most common form of nitrogenase is the iron-molybdenum form that is composed of dinitrogenase (MoFe protein or NifDK) and dinitrogenase reductase (Fe protein or NifH) (Dixon and Kahn, 2004).

The reduction of one N_2 to two NH_3 by nitrogenase requires the hydrolysis of at least 16 ATP molecules. In order to optimize the use of energy and fixed nitrogen, most diazotrophs can shut down or turn-on the nitrogen-fixation system according to the availability of nitrogen sources in the environment. Nitrogenase regulation can occur at both transcriptional and posttranslational levels (Dixon and Kahn, 2004; Huergo et al., 2012).

The transcriptional activator NifA coordinates the transcription of the *nif* genes in Proteobacteria (Dixon and Kahn, 2004). In *A. brasilense*, NifA is constitutively expressed (Fadel-Picheth et al., 1999), but the protein is inactive under conditions of excess O_2 or NH_4^+ (Arsene et al., 1996). Activation of NifA upon removal of NH_4^+ requires the signal transduction P_{II} protein GlnB (Arsene et al., 1996; Araujo et al., 2004; Sotomaior et al., 2012). A conserved NifA motif containing four cysteines is likely to be involved in the inactivation of NifA under high oxygen levels (Dixon and Kahn, 2004).

13.2 REVERSIBLE ADP-RIBOSYLATION REGULATES NITROGENASE ACTIVITY IN *A. brasilense* AT A POSTTRANSLATIONAL LEVEL

Once synthesized, the nitrogenase enzyme of *A. brasilense*, as well as of some other diazotrophs, is reversibly inactivated *in vivo* in response to the availability of NH_4^+ (Huergo et al., 2012). In *A. brasilense*, this inactivation

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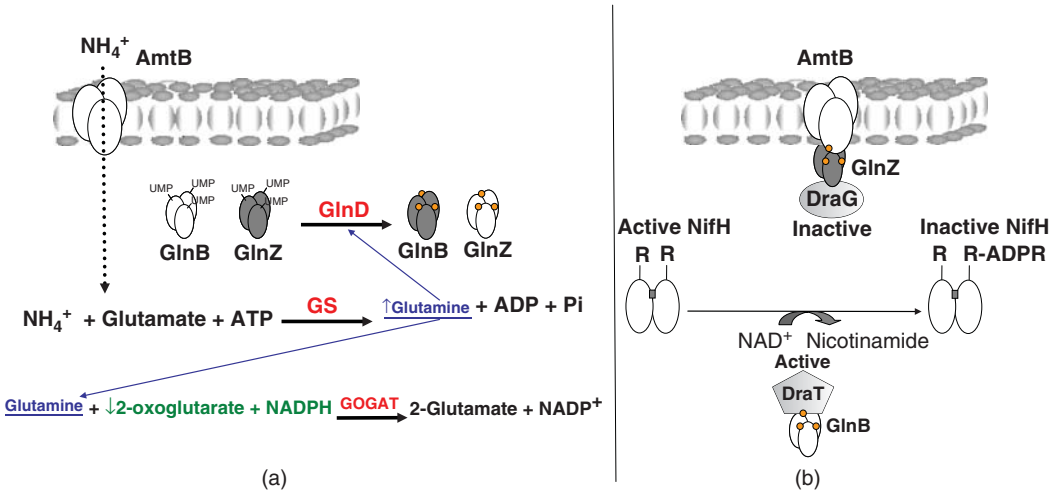

Figure 13.1 Role of the P_{II} proteins in the regulation of nitrogenase activity in *A. brasilense*. (a) When nitrogen-limited cells find ammonium in the external media, NH_4^+ is assimilated through the GS/GOGAT pathway causing an increase in the intracellular glutamine and a decrease in the 2-oxoglutarate. Glutamine binds to the GlnD enzyme, favoring the removal of UMP groups from the P_{II} (GlnB and GlnZ) T-loops. This decrease in the 2-oxoglutarate facilitates the binding of ADP (represent by orange dots) to P_{II}. (b) Few seconds after the ammonium shock, DraT is activated by the interaction with GlnB-promoting NifH-ADP-ribosylation and switching off nitrogenase activity. At the same time, DraG is inactivated by membrane sequestration through the formation of the AmtB–GlnZ–DraG ternary complex on the membrane.

involves ADP-ribosylation of the Fe-protein (dinitrogenase reductase NifH) that is catalyzed by dinitrogenase reductase ADP-ribosyltransferase (DraT) (Fig. 13.1b). Nitrogenase inactivation is reversed, after consumption of NH_4^+ by the cellular metabolism, through the action of dinitrogenase reductase activating glycohydrolase (DraG) (Huergo et al., 2012).

The activities of both DraT and DraG are oppositely regulated according to the levels of ammonium. Under nitrogen-fixation conditions, DraT is inactive and DraG is active. When external ammonium becomes available, DraT is activated while DraG is inactivated (Huergo et al., 2012). The activities of both DraT and DraG are regulated through interactions with P_{II} signal transduction proteins (Fig. 13.1; see also Chapters 11, 12).

Nitrogenase can also be ADP-ribosylated under low energy conditions such as anaerobiosis (in the organotroph *A. brasilense*) or darkness (in the phototroph *Rhodospirillum rubrum*; see Chapter 12). When oxygen or light return to optimum levels, the ADP-ribosyl attached to nitrogenase is removed by DraG. The mechanism that regulates DraT and DraG in response to the energy (oxygen or light) is still elusive. In contrast, much more is known about the ammonium signaling pathway. In the latter case, the P_{II} signaling proteins regulate DraT and DraG reciprocally (Huergo et al., 2012).

13.3 P_{II} PROTEINS REGULATE DraT AND DraG ACTIVITY

The P_{II} proteins constitute a family of highly conserved trimeric proteins that regulate the nitrogen and carbon metabolism in prokaryotes and plants (Huergo et al., 2013). P_{II} proteins can bind ATP, ADP, and 2-oxoglutarate (2-OG), and these molecules are called "P_{II} effectors" (Huergo et al., 2013). ATP and ADP bind to the same site at the interface between two subunits of P_{II} trimers in a competitive manner and with different affinities (Jiang and Ninfa, 2009). Each P_{II} trimer can bind up to three adenine nucleotide molecules; therefore, there are different possible combinations of bound ATP/ADP for P_{II} trimers (da Rocha et al., 2013). The three 2-OG binding sites are also located in the lateral clefts between P_{II} subunits, and 2-OG binding is dependent on the presence of bound MgATP (Truan et al., 2010; Fokina et al., 2010) (Fig. 13.2).

The effector ligands affect the P_{II} structure mainly at the base of a long, flexible loop known as "the T-loop" (Fig. 13.2). As a result, the energy landscape of T-loop conformations is differently affected by the different ligands modulating the ability of P_{II} to interact (through The T-loop) with various target proteins including enzymes, transcriptional factors, and transporters (Huergo et al., 2013). In some prokaryotes, the possible T-loop conformations and interactions are also influenced by covalent posttranslational modification of some of its residues.

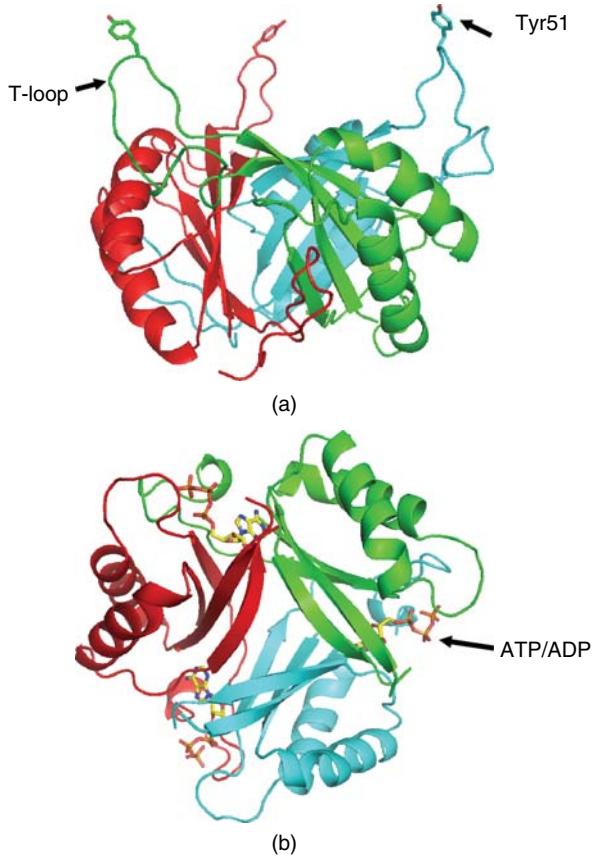

Figure 13.2 Characteristic structural elements the P_{II} proteins. (a) The arrows indicate the T-loop and the conserved Tyr51 residue. (b) The arrow indicates the lateral clefts between each P_{II} subunit where ATP and ADP bind competitively. (*Source*: From Huergo et al., FEMS Microbiol. Rev. 2013 37(2):251–283.)

Two P_{II} proteins, namely GlnB and GlnZ, are encoded by the *A. brasilense* genome (de Zamaroczy et al., 1996). Under nitrogen-limiting conditions, each monomer of GlnB and GlnZ is covalently modified by uridylylation of the T-loop residue Tyr51 (Fig. 13.1a). When ammonium is available in the environment, GlnB and GlnZ are de-uridylylated by GlnD, a bifunctional enzyme catalyzing the uridylylation/de-uridylylation process (Araujo et al., 2008). The switch between the GlnD opposing enzymatic activities is dictated by the intracellular availability of glutamine which, in turn, depends on the environmental levels of ammonium (Fig. 13.1a; see also Chapters 11, 12).

In vivo pull-down experiments in *A. brasilense* revealed physical interactions between DraT–GlnB and DraG–GlnZ, suggesting that DraT and DraG activities are controlled by interactions with the P_{II} proteins (Huergo et al., 2006a, b). The P_{II} effectors and uridylylation affect the complex formation between DraT–GlnB and DraG–GlnZ *in vitro* (Huergo et al., 2012). Both complexes are more stable when the P_{II} proteins are de-uridylylated (high nitrogen level) and when the ATP/ADP ratio and the 2-OG levels are low (Huergo et al., 2012). A decrease in the 2-OG levels signals abundance of available fixed nitrogen in Prokaryotes (Dodsworth et al., 2005; Yuan et al., 2009; Radchenko et al., 2010); therefore, the DraT–GlnB and DraG–GlnZ complexes are stabilized when the ammonium supply is abundant, that is, high glutamine and low 2-OG (Fig. 13.1a). The interaction between DraT and GlnB leads to DraT activation *in vitro*, promoting NifH ADP-ribosylation and thus nitrogenase inactivation when ammonium is sufficiently available in the environment (Moure et al., 2013). In order to avoid a futile cycle of nitrogenase ADP-ribosylation, DraG must be inactivated upon DraT activation. Hence, complex formation between DraG and GlnZ in response to increased ammonium and thus DraT activation must result in DraG inactivation. In order to understand how the interaction between DraG and GlnZ may affect DraG activity, the X-ray structure of the DraG–GlnZ complex was determined in the presence of ADP at 2.1 Å.

In contrast to all other known P_{II}–target protein complex studies, so far the DraG–GlnZ complex does not depend on the formation of contacts involving the T-loop. Indeed, a GlnZ variant carrying a T-loop deletion is still able to interact with DraG (Rajendran et al., 2011). Instead, a lateral surface region of the trimer comprising two GlnZ monomers is the site for DraG interaction (Fig. 13.3).

Comparison between the structures of GlnZ bound to Mg-ATP/2-OG and the structure of GlnZ bound to ADP within the DraG–GlnZ complex explains how increased 2-OG can negatively affect the DraG–GlnZ interaction. With Mg-ATP/2-OG bound, the GlnZ T-loops extend at nearly 90° to the GlnZ trimer axis, and this conformation would severely clash with bound DraG. By contrast, with bound ADP the largely disordered GlnZ T-loops appear to extend more parallel to the trimer axis and therefore avoid clashing into bound DraG (Fig. 13.4). On the other hand, comparison of the DraG structure with and without bound GlnZ does not show any significant conformational changes in DraG upon interaction with GlnZ. DraG activity, when assayed using a low molecular mass of artificial substrate *in vitro*, is unaffected in the presence of GlnZ (Li et al., 2009; Rajendran et al., 2011), indicating that the interaction with GlnZ does not affect the catalytic machinery nor prevent access of this low molecular mass substrate to the active site of DraG. *In vivo* DraG inactivation in response to ammonium is therefore not simply explained by GlnZ complex formation and requires additional considerations.

Experimental data indicate that a critical component for DraG inactivation is the ammonium transporter protein AmtB (Huergo et al., 2006b). The AmtB protein is an integral membrane protein that forms a pore to conduct ammonia across the cell membrane (Zheng et al., 2004; Fig. 13.1a). When the *amtB* gene of *A. brasilense* is disrupted, the cells fail to ADP-ribosylate nitrogenase but are unaffected in their

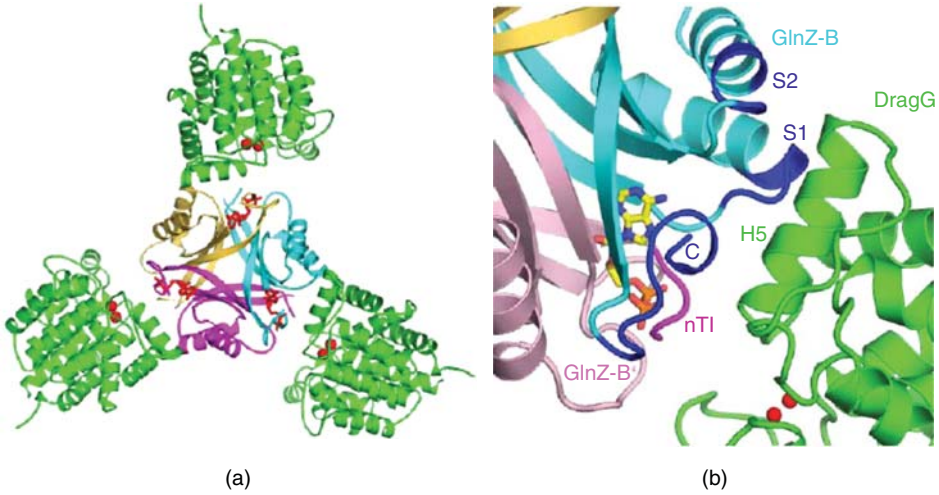

Figure 13.3 Structure of the trimeric *A. brasilense* DraG–GlnZ complex. (a) Cartoon representation of three DraG monomers (green) bound to the central GlnZ trimer (cyan, light pink, and orange). The ADP molecules bound in the lateral clefts between GlnZ chains are shown as red sticks, the two Mg^{2+} ions are present in the active site of each DraG monomer as red spheres. (b) Closeup of the DraG–GlnZ interface involving the two GlnZ monomers B and B′ (cyan and magenta, respectively). The DraG-interacting chain segments S1, S2, and C of monomer GlnZ-B are emphasized in dark blue; the N-terminal part of the interacting T-loop of chain GlnZ-B′ in magenta, and ADP is shown in yellow sticks. (*Source*: Figure 1 from Rajendran C PNAS 2011 108(47):18972–6.)

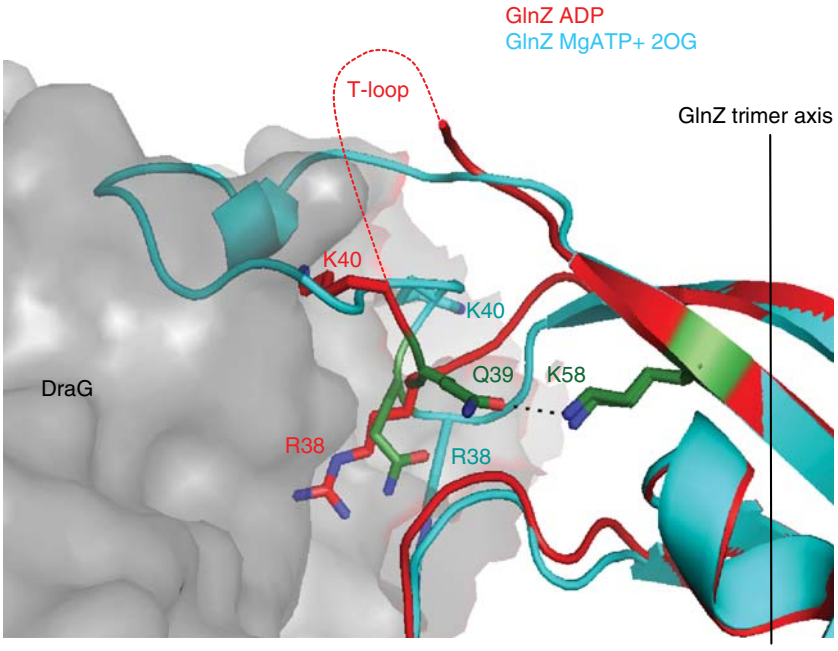

Figure 13.4 Comparison between the structures GlnZ bound to Mg-ATP and 2-OG, and the structure of GlnZ bound to ADP within the DraG–GlnZ complex. The structure of GlnZ bound to MgATP and 2-OG (cyan) was superimposed to the structure of the GlnZ bound to ADP within the DraG–GlnZ complex (red). The DraG surface is shown in gray. Key GlnZ residues that stabilize the different GlnZ T-loop conformations are indicated by sticks. The part of the GlnZ T-loop that was not resolved in the DraG–GlnZ is represented by a dashed red line.

capacity to take up ammonium from the media. The failure of the *amtB* mutant to modify nitrogenase correlates with defects in DraG inactivation rather than DraT activation (Huergo et al., 2006b). Interestingly, the GlnZ–DraG complex is still formed in the *amtB* mutant background (Huergo et al., 2006a), again supporting the fact that the GlnZ–DraG interaction is not sufficient for DraG inactivation.

The AmtB protein is the best described protein whose activity is regulated by the interaction with P_{II} proteins. When the ammonium level rises, there is a decrease in the intracellular 2-OG, which facilitates the occupancy of the P_{II} trimer by ADP (Radchenko et al., 2010; Gerhardt et al., 2012; da Rocha et al., 2013). Bound ADP stabilizes a P_{II} T-loop conformation that can bind at the cytoplasmic face of

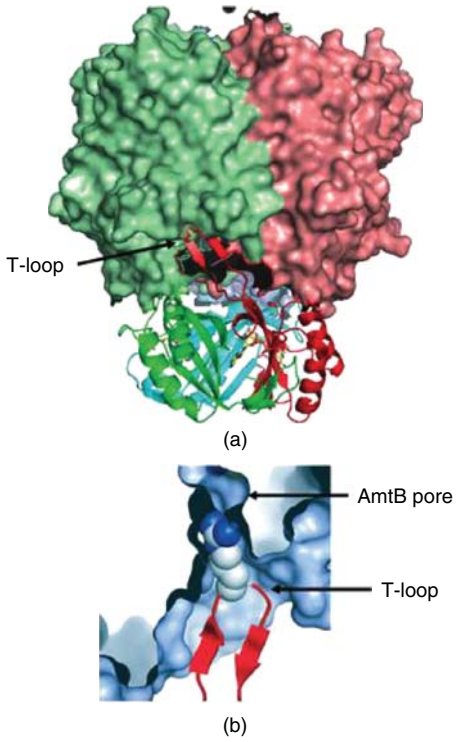

Figure 13.5 The *E. coli* AmtB–GlnK complex. (a) Overview of the complex. (b) Detail view of T-loop insertion of AmtB cytoplasmic channel exit. (*Source*: Figure 1B/Figure 5C from Conroy MJ et al. PNAS 2007, 104(4):1213–8.)

the ammonia pore of AmtB blocking the transporter activity (Coutts et al., 2002; Javelle et al., 2004; Conroy et al., 2007; Rodrigues et al., 2011) (Fig. 13.5).

In vitro analysis showed that in *A. brasilense*, the AmtB, GlnZ, and DraG proteins can form a ternary complex in the presence of ADP (Huergo et al., 2007). This is supported by *in vivo* data showing that DraG binds to the membrane in a GlnZ–AmtB dependent manner when the ammonium levels rise (Huergo et al., 2006b). Indeed, the AmtB–GlnZ–DraG ternary complex can be easily modeled based on the *Escherichia coli* AmtB–GlnK and *A. brasilense* GlnZ–DraG structures without structural clashes (Fig. 13.6).

The model structure of the AmtB–GlnZ–DraG complex clearly shows that the ADP-ribosylated NifH would not be able to access DraG, causing DraG inactivation by steric hindrance of its active site (Fig. 13.6). However, this also holds true for the GlnZ–DraG binary complex; a structural model of ADP-ribosylated NifH cannot be accommodated in the DraG active site without severe clashes between the NifH and GlnZ structures (Fig. 13.6) (Rajendran et al., 2011; Huergo et al., 2006a). If DraG–GlnZ complex formation, known to occur after an ammonium pulse in this genetic background, were sufficient to inactivate DraG, how could the *amtB* mutant fail to inactivate DraG?

The answer to this question may be related to the flexibility of the GlnZ T-loop. In the DraG–GlnZ complex, the GlnZ T-loop remains rather mobile as it cannot be seen in the electron density (Fig. 13.5). The intrinsic mobility of the GlnZ T-loop would be further restricted upon interaction with DraG imposing an entropic penalty on the formation of the DraG–GlnZ complex. Indeed, a GlnZ variant carrying a T-loop deletion binds more avidly to DraG. This is further supported by calorimetric analysis of the DraG–GlnZ complex (Rajendran et al., 2011) that shows a positive value for the term $-T\Delta S$ in the Gibbs free energy equation. As a result, the complex between DraG and GlnZ has an overall modest affinity (1.5 μM) and ADP-ribosylated NifH may be able to out-compete GlnZ for DraG binding, allowing

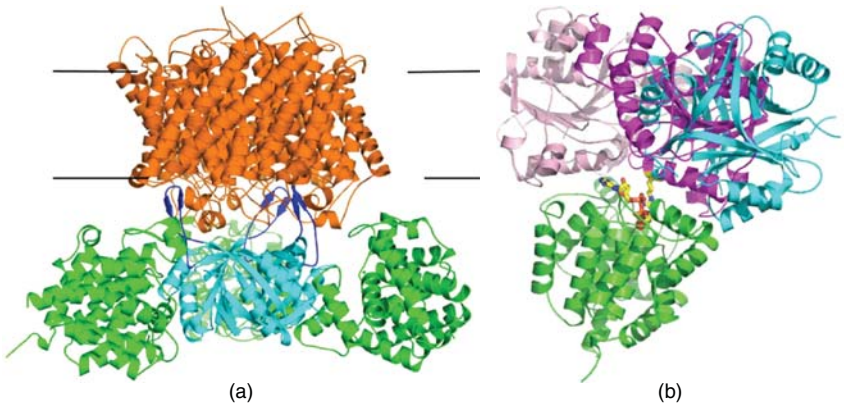

Figure 13.6 Cartoon representation of a putative *A. brasilense* AmtB–DraG–GlnZ ternary complex and a modeled DraG–NifH complex. (a) The *E. coli* AmtB (orange)–GlnK (cyan) complex (2NUU) was superimposed on the *A. brasilense* complex of DraG (green) with GlnZ (not shown for clarity) using their structurally very similar P_{II} trimers. The (ordered) GlnK T-loops are emphasized in dark blue. (b) Illustration of the steric overlap between the DraG (green)–GlnZ (cyan) complex and a modeled complex of DraG with a mono-ADP-ribosylated NifH dimer (light pink and magenta subunits). The ADP-ribosylated R101 side chain is shown in yellow stick representation, and the Mg^{2+} ion is shown at the DraG active site as a red sphere. (*Source*: Figure 4 from Rajendran C PNAS 2011 108(47):18972–6.)

the ADP-removal reaction to occur even after a rise in ammonium in an *amtB* background.

On the other hand, after an ammonium shock in the presence of AmtB, the AmtB–GlnZ–DraG complex forms and the GlnZ T-loop will be in a fixed position as it engages in interactions with the cytosolic face of AmtB (Fig. 13.6). This would reduce the entropic penalty for DraG binding such that DraG binding to AmtB-bound GlnZ would be considerably enhanced, and competition by ADP-ribosylated NifH would be more strongly prohibited.

13.4 CONCLUSIONS

The solution of the crystal structures of DraG and of the DraG–GlnZ complex has facilitated the comprehension of the nitrogenase ADP-ribosylation system in *A. brasilense.* These protein structures together with biochemical data and *in vivo* studies support a model where DraG is inactivated by the formation of a ternary complex involving DraG, the P_{II} protein GlnZ, and the ammonium transporter protein AmtB on the cell membrane (Fig. 13.1). The formation of the AmtB–GlnZ–DraG complex would impede the access of ADP-ribosylated NifH to the DraG active site, thereby inactivate DraG in response to ammonium.

The model for the regulation of DraG in *A. brasilense* presented here shares some similarities to the one proposed to operate in *R. rubrum* (Nordlund and Hogbom, 2013; see Chapter 12). In the latter organism, it is believed that both the AmtB and P_{II} participate in the inactivation of DraG in response to ammonium; this inactivation would also involve the binding of DraG to the cell membrane. However, the formation of a ternary complex between AmtB, P_{II}, and DraG has been excluded in the *R. rubrum* model. In contrast, DraG would bind to the membrane through direct contact with an unknown membrane protein (Nordlund and Hogbom, 2013).

ACKNOWLEDGMENTS

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Chapter 14

Fe Protein Overexpression Can Enhance the Nitrogenase Activity of *Azotobacter vinelandii*

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14.1 INTRODUCTION

Although biological nitrogen fixation (BNF) is widely known in bacterial species, only a few can carry out BNF under free living conditions. *Azotobacter vinelandii* is unique in its ability to fix nitrogen in aerobic free living conditions. Studies with *Azotobacter* have led to the elucidation of nitrogenase, its subunits and kinetics; see Chapter 9. Most bacteria capable of BNF have a single type of nitrogenase enzyme (Nitrogenase I) consisting of the Fe-protein and MoFe subunits (expressed from the *nifH* and *nifDK* genes, respectively) (Seefeldt et al., 2009; Robinson et al., 1986). However, *A. vinelandii* produces three types of nitrogenases under different conditions: Nitrogenase I is expressed under normal conditions; Nitrogenase II consists of the Fe- and VnFe-protein subunits under Mo-limiting conditions (Joerger et al., 1990); and Nitrogenase III consists only Fe in its dinitrogenase and dinitrogenase reductase subunits under Mo- and Vn-limiting conditions (Hales et al., 1986). Nitrogen fixation is a highly energy-dependent process and is also highly sensitive to oxidative conditions and the nutritional status of the cell. Nitrogenase I, which is most widely studied, is stringently regulated at the transcriptional level by NifA (enhancer-binding protein) and NifL (regulatory protein) with the help of Sigma54 (RNA polymerase) (Dixon and Kahn, 2004).

Nitrogenase-mediated BNF requires 16 molecules of MgATP for each molecule of NH_3 produced (Tezcan et al., 2005). Because of the high-energy requirement and slow turnover of Nitrogenase I (Dixon and Kahn, 2004), *Azotobacter* fixes nitrogen sufficient for its own needs only. It releases very small amounts of ammonium into the environment under favorable conditions, releasing most of the ammonium after death and lysis (Bali et al., 1992). Studies relating to the improvement of the nitrogen-fixing ability of diazotrophs have been focused on either physiological suppression of nitrogen assimilatory genes (Bali et al., 1992) or creating mutations and selecting higher nitrogen-fixing mutants (Gordon and Moore, 1981). Attempts to increase the NifA production in order to increase nitrogen-fixation efficiency (NFE) have revealed that complete deregulation of the system leads to the loss of survivability of the cells (Bali et al., 1992; Brewin et al., 1999). Hence, increasing the NFE in a controlled manner may be one of the solutions. The complexity of enhancing the nitrogen-fixing efficiency can be deduced from the fact that 82 genes are involved in

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the entire process in addition to a variety of cellular housekeeping genes involved in carbohydrate metabolism, iron uptake, and oxygen stress (Dos Santos and Dean, 2011). Many of the proteins involved in the process are rich in iron (Hu and Ribbe, 2011; Raulfs et al., 2009). Moreover, the nitrogenase enzyme itself is oxygen labile. Thus, understanding the nitrogenase cycle and its physiological impact on the cell is important.

The Fe-protein subunit (encoded by *nifH* gene) of nitrogenase is central to the *A. vinelandii* nitrogenase cycle. Dinitrogenase (MoFe protein) can accept electrons only from the Fe protein (Rubio and Ludden, 2005), although the presence of other reductase-like ferrodoxins is also required (Egener et al., 2001). NifH and MgATP are also essential for the maturation of FeMo-co precursor into a fully assembled MoFe subunit (Rubio et al., 2002). The Fe protein probably acts as the Mo/homocitrate insertase of the NifEN-bound Mo-free FeMo-co precursor (Hu et al., 2006).

In our endeavor to increase the nitrogen-fixing efficiency of *A. vinelandii* (strain: UW), we chose to overexpress the *nifH* gene (Fe-protein subunit) from a heterologous promoter that can be induced. An RK2-based plasmid, pJB654, reported to exist stably in *A. vinelandii*, was selected as vector containing the *Pm* promoter, which is inducible with *meta*-toluic acid (Blatny et al., 1997). The *nifH* gene of *A. vinelandii* was inserted downstream of this promoter; and Fe-protein expression, acetylene reduction (nitrogen-fixation activity), and ammonium excretion were measured. We also assessed the amount of stress produced, as a result the higher nitrogen was fixed under induced conditions by determining the cellular levels of superoxide dismutase (SOD) and the levels of catechol were produced as a result of higher Fe uptake.

14.2 METHODS

14.2.1 Strains and Growth Conditions

Escherichia coli cells harboring plasmid pDB6 (pUC_{18} containing *A. vinelandii nifHDK* insert), pGEMT Easy (Promega), and pJB654 (broad host range expression vector containing the TOL promoter Pm and its regulator *xylS*, parDE, *oriV*, and *trfA* fragments) (Blatny et al., 1997) were grown with 50 μg ampicillin ml^{-1}. *E. coli* cells harboring plasmid RK2013 (Sia et al., 1995) were grown with 50 μg kanamycin ml^{-1}. *A. vinelandii* was grown in nitrogen-free modified Burk's medium (BN^-) (Page and Sadoff, 1976) at 30 °C with aeration and was maintained on agar plates of the same medium for up to 2 weeks. *E. coli* DH5α and HB101 were grown in Luria Bertini (LB) medium at 37 °C with aeration. Transconjugates were maintained on appropriate agar plates containing 50 μg/ml ampicillin or kanamycin. The growth characteristic of the transconjugants was compared with the parent strain UW in the presence of 4 μM *m*-toluic acid.

14.2.2 Construction of Recombinant Plasmids

The *A. vinelandii nifH* fragment (873 bp) was amplified with *Pfu*-I enzyme (Fermentas, Germany) from plasmid pDB6 (Jacobson et al., 1989) using the primers NifH1 (5′tcccc/gggatggctatgcgtcaatg3′) with a *Sma*I site and NifH2 (5′tag/gatcctagtcagacttcttcggc3′) with a *BamH*I site. The digested PCR product was subcloned into the pGEM-T Easy vector by TA cloning. The *Sma*I-*BamH*I fragment from pGEM-T-*nifH* was finally cloned into the broad host range expression vector pJB654 to generate plasmid pJB654-N. A schematic representation of the cloning steps is illustrated in Figure 14.1.

14.2.3 Conjugation of *A. vinelandii* UW with pJB654-N from DH5α

pJB654-N was transferred from *E. coli* DH5α to *A. vinelandii* UW by triparental mating using the helper plasmid pRK2013 (Ditta et al., 1980). *A. vinelandii* UW and *E. coli* DH5α carrying plasmid pJB654-N and helper *E. coli* (HB101) carrying plasmid pRK2301 were each cultured overnight at the appropriate temperatures and the cultures were adjusted

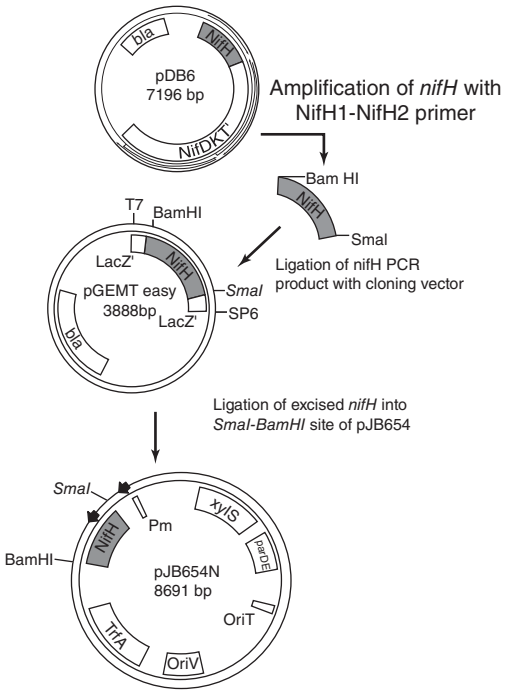

Figure 14.1 Schematic diagram showing the construction of pJB654-N. (*Source*: Adapted from Nag and Pal, 2013.)

to 10^9 cells ml^{-1}. The three types of cells were mixed in equal volumes in a 1.5-ml centrifuge tube and filtered through a 0.2-µm cellulose acetate filter membrane. The membrane was placed, bacterial side up, on a BN^- agar plate and incubated for 6–8 h at 30 °C. The cells were resuspended in 0.5-ml BN^- medium by rinsing the membrane. The transconjugates were plated, after dilutions, on BN^- agar plates with 50 µg ampicillin per milliliter. Transconjugants were restreaked four to five times on BN^- ampicillin plates in order to purify *A. vinelandii* cells harboring the plasmid pJB654-N. In order to examine the expression of the exogenous *nifH* fragment, PCR analysis was carried out with DNA extracted from the transconjugants using two sets of primers, Pm1 (5′′ggctatctctagaaaggcct3′) and NifH2.

14.2.4 Immunoblotting of NifH

Protein extraction was carried out using osmotic shock, and electrophoresis was achieved using a 10% SDS-polyacrylamide gel according to published protocols (Vázquez-laslop et al., 2001) with few modifications. Briefly, 2 ml cells were harvested, washed in an equal volume of PBS, and resuspended in a 200 µl of lysis buffer (40% glycerol in 33 mM Tris-Cl, pH 7.4 and 0.1 mM EDTA, pH 7.4). The cells were incubated at room temperature for 25–30 min and resuspended in 30 µl ice cold milli-Q water. 4 mM dithionite (DTH), 0.2 mg dithioerythritol (DTE) ml^{-1}, 20 µg lysozyme ml^{-1}, 0.1mg RNase ml^{-1}, and 0.1mg DNase ml^{-1} were sequentially added to the suspension. The mixture was incubated at room temperature for 20 min under vacuum, centrifuged at 10,000 *g*, and the supernatant was immediately used for anoxic nondenaturing acrylamide gel analysis (Allen et al., 1995). The running buffer was supplemented with 2 mM DTH, and the gel was pre-run for 45 min. Immunoblotting was carried out according to a published protocol (Sambrook et al., 1989) using an anti-*A. vinelandii* NifH antibody (Kindly supplied by Prof. P. W. Ludden, University of California, Berkeley).

14.2.5 Assay for Superoxide Dismutase

The procedure developed by Beauchamp and Fridovich (1971) was followed to assay the presence of SOD. The SOD band intensity was determined using the domain ImageJ program (Java version of NIH Image developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij/). Fluorescence intensity was measured as the area under peaks produced by NIH ImageJ.

14.2.6 Acetylene Reduction Assay

Cells were grown overnight in Burk's medium containing 29 mM NH_4Cl, harvested and washed with fresh ammonium-free medium, subcultured in the same ammonium-free medium, and incubated for the desired time (until log phase). The transconjugants were grown in ampicillin-containing medium and 4 µM *m*-toluic was acid added as the inducer. For acetylene reduction assay (Burris, 1972), 2 ml aliquots of *A. vinelandii* cells grown to mid-log phase were transferred to 27 ml sealed vials. About 10% of their air space was replaced with acetylene gas and incubated at 30 °C for 2–5 h. Using an airtight syringe 1 ml of gas from the head-space was injected into a Porapak N column of a gas chromatograph (model: NUCON 5765, India). The column temperature was 80 °C, and the temperature of the injector and detector was set at 120 °C. The amount of ethylene produced was calculated by the formula:

$$\text{Nanomoles of ethylene produced hour}^{-1}\text{mg}^{-1}\text{protein} = \frac{C \times P_s \times A_s \times V}{P_{std} \times A_{std} \times T \times P}.$$

where, C is the concentration of ethylene in standard; P_s is the peak height of sample; A_s is the attenuation used for sample; P_{std} is the peak height of standard; A_{std} is the attenuation used for sample; T is the time of incubation; and V is the volume of air space in the assay vial.

14.2.7 Ammonium Production Assay

The ammonium concentration in the medium was estimated as follows. Aliquots were taken at different times and *A. vinelandii* cells were spun down. Appropriate amounts of supernatant were assayed for ammonium by the indophenol method (Burris, 1972).

14.2.8 Spectrophotometric Assay for Siderophore (Catechol)

The cells were removed by centrifugation, and the culture supernatant fluid, containing the catechol released by the bacterial cells, was acidified to pH 1.8 with HCl and passed through a Millipore filter (pore size: 0.2 µm). The acidified culture fluid was scanned with a Hitachi U2800 spectrophotometer, absorbance at 310 nm being used to compare total catechol amounts (Page and Huyer, 1984).

14.3 RESULTS

The P_m-*nifH* construct introduced in the UW strain was verified by sequencing the *nifH* gene. The selected conjugant was designated UW/pJB654-N. The inducer of P_m promoter, *m*-toluic acid, is a substrate in the toluic acid pathway and was considered to be toxic to bacterial cells at high concentrations (Marques and Ramos, 1993). Therefore,

we assessed the effect of different concentrations of *m*-toluic acid on strain UW.

It was found that UW could grow in N-free medium containing *m*-toluic acid as the sole carbon source. This effect was diminished when sucrose was added to the medium, since the cells were utilizing sucrose preferentially. It was also found that the addition of the inducer improved the acetylene reduction capacity of strain UW. We used 4 μM *meta*-toluic acid for further analysis. As *m*-toluic acid alone was used by UW as a substrate, we had to normalize our analysis by growing the wild-type strain with the same concentration of the inducer. We performed uninduced control reactions as well. Immunoblots show that UW/pJB654-N produced twofold to threefold higher Fe protein at log phase (OD_{600nm}=0.9) and 1.2 fold higher Fe protein at stationary phase (OD_{600nm}=1.2) than UW cells (Fig. 14.2a). This led to higher nitrogen-fixation activity, as assessed by acetylene reduction and ammonium estimation assays. During log phase, 7.4 nM ethylene $hour^{-1}$ mg^{-1} protein was produced by UW/pJB654-N cells as compared to 2.64 nM ethylene $hour^{-1}$ mg^{-1} protein produced by UW. The acetylene reduced during stationary phase was less in both UW and UW/pJB654-N, though UW/pJB654-N (2.1 nM ethylene $hour^{-1}$ mg^{-1} protein) displayed higher acetylene reduction than UW (0.2 nM ethylene $hour^{-1}$ mg^{-1} protein) (Fig. 14.2b). In uninduced experiments, 60% more Fe protein was detected in UW/pJB654-N, but only a 5% higher acetylene reduction activity than UW (results not shown). We concluded that the increase in acetylene reduction activity in the UW/pJB654-N was due to the increased amount of Fe protein produced by the plasmid, as the endogenous *nifHDK* operon continued to be driven by its own promoter in both UW and UW/pJB654-N. In the log phase, uninduced UW and UW/pJB654-N cells released comparable amounts of ammonium. However, when the cells were grown in the presence of 4 μM *m*-toluic acid, the transconjugants released 1.67-fold more ammonium than UW at log phase, whereas this difference was much higher (2.16-fold) during the stationary phase (Fig. 14.2c).

As discussed earlier nitrogen fixation produces stress inside the cells, and this may be one of the reasons why increasing the NFE is so difficult. In order to assess whether the increase in acetylene reduction activity due to increased Fe protein produced additional stress, we analyzed the most common stress-related enzyme SOD. We were surprised to observe that the SOD activity of UW were higher than UW/pJB654-N in the log phase. The SOD activity of UW was approximately 25% higher than that of UW/pJB654-N. However, during the stationary phase, UW/pJB654-N expressed 70% more SOD as compared to UW. The uninduced control experiments showed almost no difference in SOD production between UW/pJB654-N and UW during the log phase. In the stationary phase of the uninduced experiments, UW/pJB654-N had 26% more SOD content as compared to UW (Fig. 14.3a).

Catechols are produced by *A. vinelandii* for uptake of Fe (Page and Huyer, 1984). *A. vinelandii* releases four siderophores: azotochelin, aminochelin, protochelin, and azotobactin, and a pseudo-siderophore dihydrobenzoic acid (DHBA) (Page and Huyer, 1984). UW/pJB654-N produced 55% more (40% more in uninduced conditions) catechol than UW (Fig. 14.3b). It is also known that catechols are usually produced in higher amounts as a result of metal depletion, which happens during the late stationary phase in laboratory conditions. Hence, we determined the amount of catechol produced during the stationary phase. UW/pJB654-N produced 110% more catechol than UW under induced conditions compared to only 28% in uninduced controls (Fig. 14.3b).

14.4 DISCUSSION

NFE may be enhanced under conditions of higher levels of Fe protein, provided excess ATP and high concentrations of FldHQ (flavodoxin hydroquinone) are available (Lowery et al., 2006). In our study, we show that NFE can be enhanced by overexpressing the Fe protein alone. We expressed *nifH* from the *Pm* promoter, which can be induced by *m*-toluic acid. Analysis of the genome sequence of *A. vinelandii* DJ (Setubal et al., 2009) revealed the presence of all the enzymes of at least one *m*-toluic acid degradation pathway. The metabolic intermediates of the *m*-toluic acid degradation enter the Krebs cycle (Marques and Ramos, 1993). A change in the ATP/ADP ratio could lead to an increase in nitrogenase activity (Lowery et al., 2006). In order to prove that the observed twofold higher acetylene reduction by UW/pJB654-N under induction by *m*-toluic acid was solely due to the increased amount of Fe protein, we added the same amount of the inducer to UW control cells. We also observed an increase in ammonium release. As *Azotobacter* utilizes ammonium for its growth and generally excretes little amounts of ammonium into their surroundings till their death and lysis (Bali et al., 1992), it was not a surprise when the amount of ammonium released by UW/pJB654-N was less (1.67-fold) as compared to the acetylene reduced (twofold higher). However, UW/pJB654-N released high amounts of ammonium during the stationary phase as expected.

Several pathways by which *A. vinelandii* protects nitrogenase from the irreversible oxidation of its components have been proposed (Maier and Moshiri, 2000). An interesting mechanism for the protection of nitrogenase is autoprotection, which involves the SOD and catalase/peroxidase enzymes of the cell. Fe protein could reduce oxygen to produce an O^- radicle and H_2O_2 (Thorneley and Ashby, 1989). These products are removed by

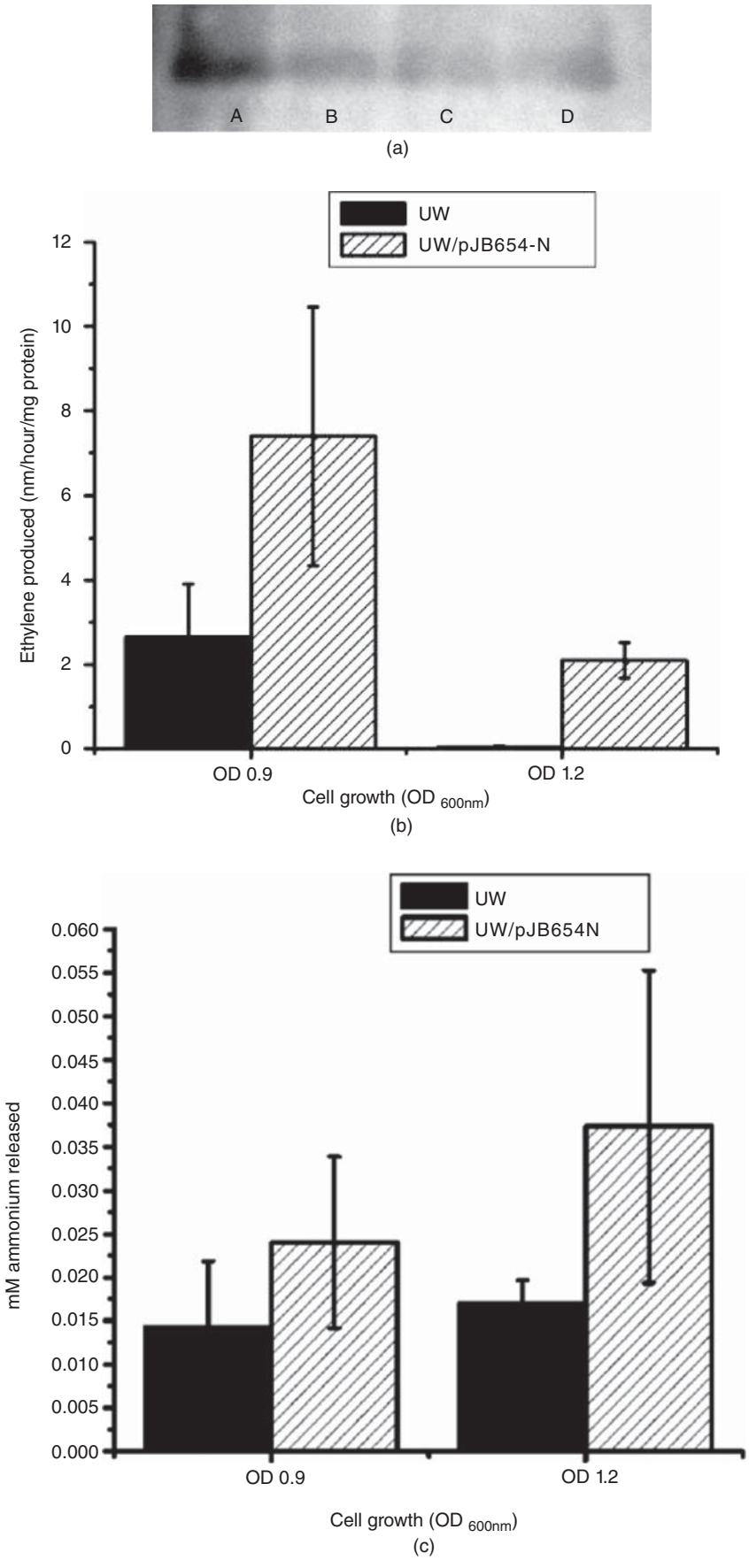

Figure 14.2 (a) Representative Western blot of UW and transformant with anti-NifH antibody induced with 4μM *m*-toluic acid. Same amounts of crude extract were loaded in each lane. Lane A: UW/pJB654-N (0.8 OD_{600}), Lane B: UW (0.8 OD_{600}), Lane C: UW/pJB654-N (1.2 OD_{600}), and Lane D: UW (1.2 OD_{600}). (b) Acetylene reduction activities of UW and UW/pJB654-N in log phase (OD_{600} = 0.8) and stationary phase (OD_{600} = 1.2). (c) Ammonium released into the medium by UW and UW/pJB654-N at mid-log phase (0.9 OD_{600}) and stationary phase (1.2 OD_{600}). (*Source*: Adapted from Nag and Pal, 2013.)

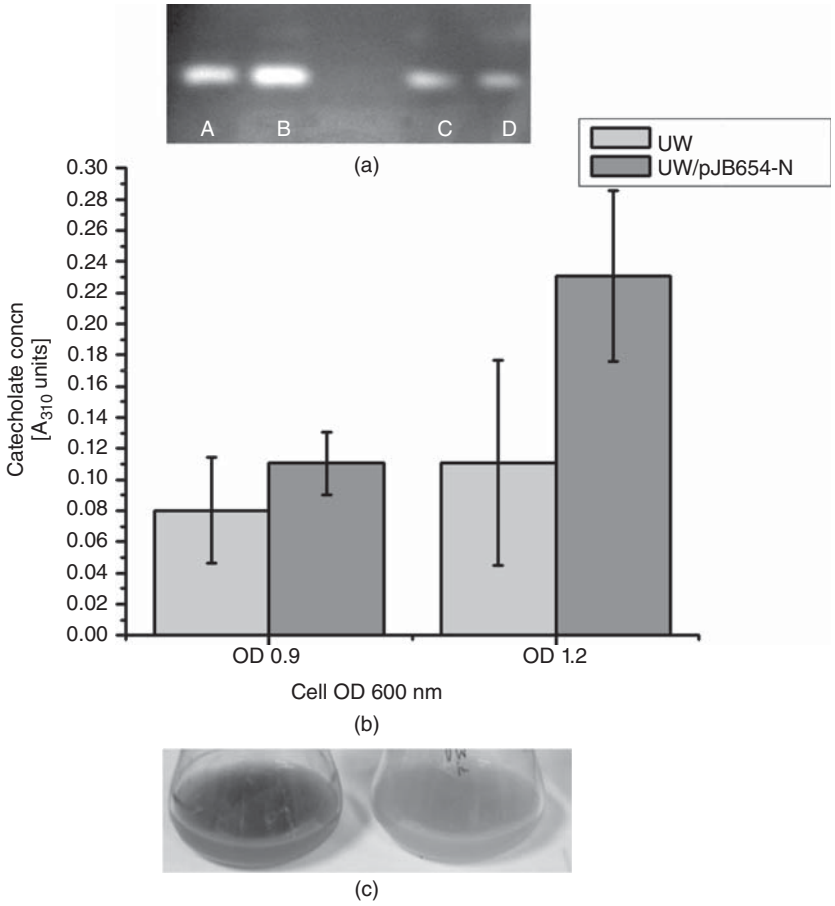

Figure 14.3 (a) Single band of Fe-SOD in 10% acrylamide gel. Lanes A, C: UW/pJB654N, Lanes B, D: UW. Lanes A, B are cells grown to OD_{600} of 0.75; and Lanes C, D are cells grown to OD_{600} of 1.8. (b) Total catechol (OD_{310}) production by UW and UW/pJB654N. Data represent mean and standard error of two independent experiments with three replicates each. (c) Production of pigments by UW/pJB654N (left) and UW (right) at stationary phase after induction. Addition of *m*-toluic acid in Burk's N-free medium may provide an advantage to the cells by acting as an additional carbohydrate substrate. (*Source*: Adapted from Nag and Pal, 2013.)

SOD and catalase/ peroxides. It has been also suggested that SOD is important in the process of nitrogen fixation as evidenced by the higher levels of SOD in nitrogen-fixing cells (Becana and Rodríguez-Barrueco, 2006; Boogerd et al., 1998). We observed that under induced conditions in the log phase (OD_{600nm} = 0.75), SOD activity of UW was about 25% higher than that of UW/pJB654-N. However, at the stationary phase (OD_{600nm} = 1.20), SOD activity of UW was depleted faster than that of UW/pJB654-N to about 70% of the latter. This result correlates with the Fe-protein production by the UW/pJB654-N and UW strains. SOD levels of cells fixing nitrogen are higher than nonfixing cells (Maier and Moshiri, 2000; Dingler and oelze, 1987). UW cells displayed SOD levels 25% higher as compared to UW/pJB654-N, even though the latter had twofold to threefold higher NFE. However, UW/pJB654-N expressed 70% more SOD than UW during the early stationary phase.

Siderophores are low molecular weight derivatives of catechols or hydroxamiic acid released by bacterial cells. Siderophores help in the solubilization, chelation, and transport of iron into the cell (Neilands, 1995). Nitrogen fixation in *A. vinelandii* is sensitive to iron. It was found to be growth-limiting for the organism at a concentration as high as 12.5 μM (Fekete et al., 1983). From our results, the enhanced total catechol production in the recombinant cells is consistent with the higher requirement of iron for the nitrogenase activity of *A. vinelandii* (Tindale et al., 2000). The production of catechols is higher during early stationary phase, when the growth medium is depleted of iron. We also observed the formation of a dark color pigment during the late stationary phase (Fig. 14.3c). Similar to the results of Shivprasad and Page (1989), formation of this dark colored pigment was optimal in the stationary phase, and there was no correlation between nitrogenase activity and pigmentation (results not shown).

14.5 CONCLUSION

Our results have shown that increasing the level of Fe protein in the cell can enhance the nitrogen-fixation activity of *A. vinelandii*. To our knowledge, this is the first report of effectively increasing the nitrogenase activity by over-expressing only one of the subunits of the enzyme. This approach may be less toxic for the cell as indicated by the

SOD activity during the exponential phase of cell growth. It would be worthwhile to explore in future if engineering the relevant metabolic pathways for ATP production, SOD expression, and iron uptake can further lead to *Azotobacter* strains with enhanced nitrogenase activity.

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Chapter 15

FNR-Like Proteins in Rhizobia: Past and Future

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15.1 INTRODUCTION

Nitrogen fixation performed by symbiotic systems requires concerted gene expression in both partners. Many regulatory circuits operate inside the nodule to carry out both, bacterial differentiation into nitrogen-fixing bacteroids and expression of *nif* and *fix* genes where low-oxygen concentration operates as the most important regulatory signal. The limited oxygen atmosphere inside the nodule is the optimal environment for the nitrogenase to reduce dinitrogen to ammonia. However, metabolic adjustments are needed to optimize oxygen consumption in respiration under this condition. The requirement of a low-oxygen environment for nitrogen fixation and the demand for optimal respiration to produce ATP seem to be a paradoxical situation in nitrogen-fixing bacteria (Dixon and Kahn, 2004; Fischer, 1994; Udvardi and Poole, 2013). Inside the nodules, oxygen diffuses by binding reversibly to leghemoglobin, and the bacteroid respiration is made possible by the expression of a cbb_3-type terminal oxidase with high affinity for oxygen. This specialized cytochrome oxidase complex is encoded by the oxygen-regulated *fixNOQP* operon (Mandon et al., 1994; Preisig et al., 1993). Therefore, a detection-effector system that responds to changes in the oxygen level is necessary to activate transcription of these genes.

Most rhizobial genes involved in nitrogen fixation are integrated in two different networks in a species-specific manner (Fischer, 1994; Kaminski et al., 1998; Udvardi and Poole, 2013). One includes the NifA and RpoN regulators, while FixL, FixJ, and FixK comprise the other regulatory cascade, specific for symbiotic diazotrophs (Dixon and Kahn, 2004; Udvardi and Poole, 2013).

FixL and FixJ are members of the two-component regulatory systems, the main mechanism employed by bacteria to coordinate their responses to environmental changes. In general, FixL is a hemoprotein with histidine kinase activity able to sense oxygen concentration. When oxygen concentration drops, the redox state of the protoporphyrin in its heme domain changes and promotes the autophosphorylation of the histidine kinase domain. Once autophosphorylation occurs, the transphosphorylation of the FixJ receiver domain can take place (Agron et al., 1993). In these model systems, FixJ is a positive regulator of *fixK*, which is a second transcriptional regulator in the regulatory cascade in response to microaerobic conditions (Hertig et al., 1989; Anthamatten et al., 1992). FixK belongs to the cyclic AMP receptor protein/fumarate and nitrate reduction regulator (CRP/FNR) superfamily of transcriptional regulators (Fischer, 1994). The CRP/FNR regulators are key distributors of a variety of environmental signals, and some

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of them are recognized as kaniglobal regulators (Körner et al., 2003). CRP and FNR from *Escherichia coli* are the two most well-studied members. CRP is a global regulator that is activated in the presence of the second messenger cAMP. CRP or CAP (catabolite activator protein) participates in the catabolic repression phenomena in *E. coli*, regulating the transcription of catabolite-sensitive genes by binding to DNA (Körner et al., 2003). The three dimensional structure of CRP and the CRP–DNA complex has been resolved and analyzed (Schultz et al., 1991; Parkinson et al., 1996a, b; Passner and Steitz, 1997). The CRP–DNA-binding site is a 22-bp palindromic sequence with the consensus sequence: AAATGTGATCTAGATCACATTT (Busby and Ebright, 1999). Fnr contains an O_2-sensitive $[4Fe–4S]^{2+}$ cluster that directly senses oxygen. In *E. coli*, it controls the expression of up to 100 genes in response to oxygen limitation (Kang et al., 2005; Kiley and Beinert, 2003). Under aerobic conditions, FNR is a monomer unable to activate transcription of its targets. However, in anaerobic conditions, the protein dimerizes and specifically binds to an imperfect palindrome known as the anaerobox [TTGATN4ATCAA] to activate or repress transcription of its regulatory targets (Spiro and Guest, 1987; Nees et al., 1988). During anaerobic conditions, each FNR monomer possesses an iron–sulfur cluster [4Fe–4S] coordinated by conserved cysteines in the N-terminal domain. The presence of iron–sulfur clusters [4Fe–4S] allows oxygen sensing; oxidation of the metal cluster promotes its disassembly and the formation of the apoprotein in aerobic conditions (Jervis and Green, 2007; Crack et al., 2008). Other important residues within the dimerization interface and near the cysteine cluster that regulates the dimer formation are serine 24 and aspartate 154 (Jervis et al., 2009; Moore and Kiley, 2001). The FNR tertiary structure has not been determined. Because of their sequence similarity, CRP has been used as template for structure modeling of FNR (Cherfils et al., 1989).

Similar to CRP targets, FNR-dependent promoters could be of three classes, named by analogy with the CRP promoters by the number and the position of the anaerobox. Class I is characterized for the presence of the FNR-binding site located near position −62. The FNR DNA-binding site in class II promoters is centered near position −41.5 overlapping the −35 box. Class III promoters have more than one FNR binding site that can be located in a class I– class I or class I–class II manner (Busby and Ebright, 1999). Three different activating regions (ARs) have been studied genetically and biochemically in *E. coli* FNR. AR1 comprises residues 71–75, 116–121, and 184–192 located in three adjacent exposed loops (Bell and Busby, 1994; Williams et al., 1997; Li et al., 1998). This structural region makes contact with the C-terminal domain of the RNA polymerase α subunit (αCTD) (Lee et al., 2000). The presence of an AR2 was uncovered by the analysis of FNR mutants that recovered activity despite the absence of a conventional AR1, in a genetic background where *rpoA* is affected or in mutants defective for iron–sulfur cluster assembly. Based on these studies, the role of residues 49 and 50 in AR2 was determined (Li et al., 1998; Ralph et al., 2001; Blake et al., 2002). Residues from 80 to 89 constitute the AR3 within a loop; this region is responsible for contacting the σ^{70} CTD, and in addition to the other ARs is crucial for transcription regulation at class II promoters. In the case of class I promoters, only AR1 is required for transcriptional regulation (Lamberg et al., 2002).

In Rhizobiales, different kinds of FNR-like proteins have been identified and grouped in three main subfamilies: FixK, FnrN, and NnrR (Körner et al., 2003). The study of these regulators has focused mainly on the identification of their regulatory targets and in some cases defining the DNA-binding site. Not too much attention has been paid to the regulatory mechanisms governing their activity. A notable exception is the regulator $FixK_2$ from *Bradyrhizobium japonicum* where studies on the biochemical properties of the purified protein have been reported (Mesa et al., 2005, 2009).

Interestingly, the FixK protein does not conserve the cysteines necessary to coordinate the iron–sulfur clusters in FNR. This particularity renders the protein unable to directly respond to the presence of oxygen. However, the role of FixK as a key distributor of the microaerobic response in rhizobia resembles the role of its homolog in *E. coli* that is required to switch from an aerobic lifestyle to an anaerobic one. Worth noting is the observation that the FixK target *fixN*, which encodes the subunit I of the cytochrome cbb_3 oxidase, has an anaerobox in its regulatory sequence (Batut et al., 1989). It has also been proposed based on model building of Fnr and FixK from *E. coli* and *Sinorhizobium meliloti*, respectively, that both regulators might bind the same DNA consensus sequence (Cherfils et al., 1989). More recent studies in *S. meliloti* and *B. japonicum* have explored the regulatory landscape of the FixK regulators in response to oxygen limitation, both under free living and symbiotic conditions. The presence of a conserved anaerobox is important for the recognition of direct targets for these regulators (Bobik et al., 2006; Mesa et al., 2008). The recognition of practically the same DNA sequences by Fnr and FixK proteins is attributed to the presence of the conserved motif EXXSR in the DNA-binding domain of both regulators (Lazazzera et al., 1993).

After the initial identification and characterization of the FixK regulator, another Fnr homolog has been found in *Rhizobium leguminosarum* bv. viciae. This protein was named FnrN and in contrast with FixK, this regulator is able to respond directly to oxygen limitation (Colonna-Romano et al., 1990; Schlüter et al., 1992). FnrN regulators conserve most of the cysteines that are important for oxygen sensing in Fnr of *E. coli*; however, their spacing in the N-terminal

motif is slightly different (Trageser and Unden, 1989; Khoroshilova et al., 1995).

Another Fnr-like regulator was found to integrate the low oxygen and N-oxides signal in *B. japonicum*. This protein belongs to the subfamily of NnrR regulators (Tosques et al., 1996; van Spanning et al., 1999). These regulators are responsible for the induction of the *nir* and *nor* genes, which encode important proteins involved in nitrogen oxide metabolism, in response to the presence of nitric oxide or chemically related species. The role of NnrR regulators in rhizobial models such as *S. meliloti* and *Rhizobium etli* CFN42 has also been explored (de Bruijn et al., 2006; Meilhoc et al., 2010; Gómez-Hernández et al., 2011).

The role of two novel Fnr-like regulators has been reported in *R. etli* CFN42. These two regulators were named StoRd and StoRf because they were found to be negative regulators of the symbiotic terminal oxidase cbb_3 (Granados-Baeza et al., 2007). Along with the dawn of high-throughput sequencing technologies in this decade, several members of the Fnr/CRP regulatory family have been identified in different rhizobia (Mesa et al., 2006; Granados-Baeza et al., 2007). This observation raises interesting questions regarding the significance of incorporating these types of regulators in the evolution of global regulatory circuits. Different regulatory mechanisms have evolved within the group of the Rhizobiales that involve Fnr-like regulators, mainly for the control of nitrogen fixation and denitrification processes and probably for other unexplored metabolic circuits. Even though over the years important genetic and physiological aspects on the role of FNR-like regulators in rhizobia have been reported, intriguing observations on the mechanism of action and implications of the highly variable regulatory circuits where these regulators are immersed are still pending further analysis. Undoubtedly, this is a fertile research area for the development and assessment of new hypothesis that certainly will enrich our knowledge of rhizobial physiology.

R. etli CFN42 induces the formation of nitrogen-fixing nodules on roots of *Phaseolus vulgaris* (bean) plants. In this strain, the genomic material is distributed in one chromosome and six large plasmids (pRet42a to pRet42f) whose sizes range from 184.4 to 642.5 kb. The genome of *R. etli* CFN42 encodes six *fnr*-related genes: *fixK*f, *fnrN*ch, *fnrN*d, *nnrR*, *stoR*d, and *stoR*f (González et al., 2006). Results in our laboratory have demonstrated that in *R. etli* a complex regulatory circuit controls the expression of *fix* genes that involves at least five transcriptional regulators of the Crp/Fnr family. The goal of this chapter is to review in a critical way what we have learned in the past decade about the role of Fnr-related regulators in controlling gene expression in *R. etli* and what features are conserved or have been adapted when compared with other model rhizobia.

15.2 REGULATION OF THE *R. etli fix* GENES: AN OVERVIEW

Although the presence of homologs of the *S. meliloti fixL, fixJ*, and *fixK* genes is a common feature among the different nitrogen fixers, the connectivity among regulatory cascades, the specific regulatory role of each element, and their target genes may vary (Anthamatten and Hennecke, 1991; Anthamatten et al., 1992; Bauer et al., 1998; Fischer, 1994; Kaminski et al., 1998).

Important differences between the mechanisms of regulation of *fix* gene expression in *S. meliloti* and *B. japonicum* have been reported in other rhizobia such as *R. etli* strains CFN42 and CNPAF512 and *R. leguminosarum* bv. viciae. In this regard, our work has mainly focused on the clarification of the regulatory scheme for *fix* genes in *R. etli* CFN42. Most of the *nif* and *fix* genes identified in this strain are located on the symbiotic plasmid (pSym or pRet42d) (Girard et al., 1991; Gonzalez et al., 2006; Soberón et al., 1999). Nevertheless, a *fix* reiterated region is localized on the plasmid pRet42f, previously considered a cryptic plasmid.

Detailed expression and epistasis analysis of the regulatory elements present in each reiteration have been carried out to establish the regulatory model of *fix* genes in *R. etli* CFN42 (Girard et al., 2000). The regulatory model of *R. etli* CFN42 *fix* genes integrates the participation of genes localized in different replicons. Furthermore, this scheme shows several novel characteristics when compared with the conventional systems *S. meliloti* and *B. japonicum*. In *R. etli*, the sensor histidine kinase FixL encoded on the pSym in other rhizobial species is located on pRet42f in a region containing copies of *fixK*, *fixNOQP*, and *fixGHIS* genes. Another puzzling aspect of the regulatory cascade in *R. etli* is the absence of a *fixJ* gene, a characteristic that is shared *with R. leguminosarum* bv. viciae. Both cytochrome oxidase complexes encoded by the reiterated operons *fixNOQP* are controlled by a novel *fixL–fixK*f cascade, where the response regulator in charge of activating *fixK*f expression is FxkR (*fxkR*-dubbed *fixK* regulator), which belongs to the OmpR/PhoB superfamily (Girard et al., 1996, 2000; González et al., 2003, 2006; Zamorano-Sánchez et al., 2012).

Although expression of both *fixN*f and *fixN*d requires FixKf, these genes exhibit a differential dependence on FixL. The expression of *fixN*f is fully dependent on FixL, while expression of the reiteration indispensable for symbiotic nitrogen fixation, *fixNOQP*d, can continue at adequate levels in the absence of FixL with the participation of *R. etli* CFN42 *fnrN* homologs (López et al., 2001). Thus, microaerobic expression of the *fixNOQP*d operon in *R. etli* is subjected to the direct regulatory input of three different activators: FixKf, FnrNchr, and FnrNd (Fig. 15.1).

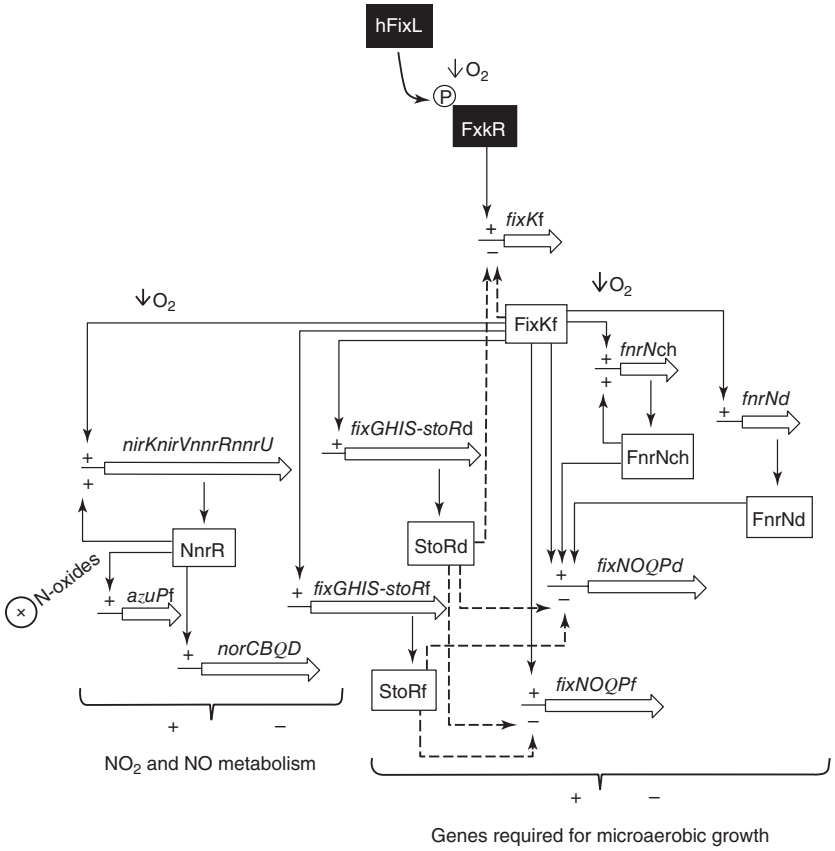

Figure 15.1 Regulatory model for *fix* gene expression in *R. etli* CFN42 under microaerobic conditions. The oxygen concentration is sensed by the two-component regulatory system hFixL-FxkR to activate FixKf, the master regulator for *fix* genes expression. The presence of N-oxides provokes the induction of the *nnrR* gene to activate the expression of genes encoding nitrite and NO-oxidases. ■ two-component regulatory system; □ FNR-like proteins; – positive regulation; --- negative regulation.

15.3 NOVEL ELEMENTS IN THE REGULATORY CASCADE OF *fix* GENES IN *Rhizobium etli*

After the release of the genome sequence of *R. etli* CFN42 in 2006 (González et al., 2006), we aimed to identify novel FNR-like regulators that might participate in nitrogen-fixation regulation. Two ORFs RHE_PD00288 and RHE_PF00499 annotated as *fnr* homologs are located downstream the *fixGHIS* operon reiterations on plasmid pRet42f and pRet42d. The genomic location of these genes suggested that they might participate in nitrogen fixation. We demonstrated that these two regulators are in an operon with *fixGHIS*, and that they operate as negative modulators of the expression of the *fixNOQP* reiterations. Negative regulation of the *fixNOQP* operons was not reported previously in model rhizobia (Fig. 15.1). These two novel reiterated regulators conserved 84% identity at the nucleotide level, and due to their role in controlling the expression of the symbiotic terminal oxidase, they were named StoRd and StoRf, respectively (Granados-Baeza et al., 2007). Apart from having very similar roles under free-living microaerobic conditions, StoRd and StoRf mutants behave different under symbiotic conditions. The physiologic studies of nitrogen fixation in *P. vulgaris* plants revealed that a mutant in StoRd produces more cbb_3 in nodules and had almost double nitrogenase activity and a 45% increase in total nitrogen content at 45 days after inoculation. On the other hand, the StoRf mutant was modestly impaired for nitrogen fixation and did not promote the same increase in cbb_3 in nodules (Granados-Baeza et al., 2007).

In *R. leguminosarum* bv. viciae, the expression of the *fixNOQP* reiterations under free living and symbiotic conditions depends on FixL and FnrN while FixK is only marginally involved (Schlüter et al., 1997). FixL, FnrN, and FixK proteins are highly conserved in *R. etli* when compared with their counterparts in *R. leguminosarum*; however, in this particular genetic background, FixKf is fundamental for the expression of *fixNOQP* genes under free living conditions. Nevertheless, under symbiotic conditions, the responsibility of promoting *fix* genes expression can rely on FixKf or on the FnrNch and FnrNd regulators (Girard et al., 2000; Lopez et al., 2001). All this aforementioned evidence shows that even though free-living microaerobic conditions have been helpful for the understanding of basic aspects of *fix* gene regulation, they do not mimic the nodule environment, and important differences in the regulation of *fix* genes can be observed in this particular niche. Because of this, it will be of great importance to expand the known repertoire of Fnr targets in *R. etli* during symbiosis with bean plants.

It also will be very interesting in terms of understanding the regulatory hierarchy of the FNR-like regulators, to know how frequently they are encountered together in the regulatory region of their target genes at different stages of the symbiosis. Furthermore, transcriptomic and proteomic analyses of nodules infected by strains mutated in FNR-like regulators will reveal important regulatory and metabolic networks that can limit nitrogenase activity and nitrogen assimilation.

15.4 NnrR LINKS THE LOW-OXYGEN AND N-OXIDE RESPONSE IN *Rhizobium etli*

Another conserved Fnr-like regulator indispensable for denitrification was found to integrate the low oxygen and N-oxides signal in *B. japonicum* (Mesa et al., 2003; de Bruijn et al., 2006). This protein belongs to the subfamily of NnrR regulators that control the expression of nitrite reductases and nitric oxide reductases in response to nitric oxide or chemically related species (Tosques et al., 1996; van Spanning et al., 1999).

R. etli is unable to use nitrate for respiration and does not encode a nitrate reductase gene. However, this bacterium encodes highly conserved homologs of the nitrite reductase (*nirK*) and the nitric oxide reductase complex (*norCBQD*). These genes are responsible for the generation and removal of nitric oxide in the nodules of *P. vulgaris*. The product of *norC* is required *in vivo* to detoxify NO. The balance between the activities of NirK and NorC determines the level of tolerance to $NO^{\cdot}$ in *R. etli* (Gómez-Hernández et al., 2011). Interestingly in *R. etli*, an *nnrR* homolog is part of an operon that includes *nirK*, *nirV*, and *nnrU* (*nirKV-nnrRU*). The *nirKnirV-nnrRU* and *norCBQD* operons are mainly expressed under low oxygen in *R. etli* and induced in the presence of nitrite or nitric oxide. Microaerobic expression of these genes requires the presence of FixKf while induction by nitric oxide or nitrite needs the presence of NnrR. Interestingly, the microaerobic expression of *norC* requires the presence of both FixKf and NnrR, while expression of *nirK* only requires FixKf (Gómez-Hernández et al., 2011) (Fig. 15.1). This suggests that FixKf can recognize the regulatory region of the *nirKV-nnrRU* operon and activate its expression, but cannot recognize the regulatory region of the *norCBQD* operon or activate its expression by itself. On the other hand, NnrR can recognize and activate transcription of *norCBQD* but requires the presence of nitric oxide to activate the expression of the *nirKV-nnrRU* operon. Binding affinity studies of Fixkf and NnrR to the regulatory region of these two operons will be fundamental for the understanding of site recognition and discrimination in bacteria that harbor several FNR-like regulators.

Two Fnr-binding sites (anaeroboxes), one of class II and one of class I, were found to be important for *nirK* expression. The class I box overlaps with a putative NifA-binding site and might play a role in positive and negative regulation of *nirK* expression. The class II anaerobox is an essential target site for *nirK* activation (Gómez-Hernández et al., 2011). Further experiments are needed to reveal the importance of these sequences for the induction of the *nirKV-nnrR* operon in the presence of nitric oxide. There is only one anaerobox upstream of *norC* [TTG-CGAATTAA-CAA], centered at position −43.5 bp from the transcriptional start site. However, its role in the expression and induction of the *norCBQD* operon in microaerobiosis in the presence or absence of nitric oxide has not been evaluated. Future efforts should be focus in the characterization of DNA recognition by NnrR in the presence or absence of nitric oxide.

15.5 CONSERVED FUNCTIONAL FEATURES IN FNR-RELATED PROTEINS OF *R. etli*

Important aminoacid residues for FNR activity have been reported in a few biological models. In an attempt to identify conserved aminoacids in FNR-like proteins from *R. etli*, we analyzed a multiple sequence alignment with FNR homologs for which a function had already been reported.

It is well known that cysteines are oxidizable targets that play important roles in protein oligomerization, sulfur cluster assembly, or protein function in general. As mentioned earlier, conserved cysteines in the amino terminal part of FNR homologs are important for oxygen sensing. In *R. etli*, the FnrN proteins are the only ones that conserved the cysteines required to ensemble the crucial iron–sulfur cluster. Similar to other FnrN proteins, they present a small difference in the spacing between second and third cysteines. StoRd has six cysteines while StoRf and FixKf have five cysteines. NnrR, similar to its homolog from *B. japonicum*, has no cysteines within its sequence. Cysteine residues are present along the sequence of these regulators, but none of them appeared to be highly conserved in all FNR-related proteins. It has been recently shown that Cys183 from $FixK_2$ is a target for oxidation not only *in vitro* but also *in vivo*. Oxidation of this residue inactivates $FixK_2$ (Mesa et al., 2009; Bonnet et al., 2013). This residue is in close proximity to the DNA-binding domain and is not conserved in any of the FNR-related proteins of *R. etli*. The role that these cysteines could play for the protein function is still unknown. None of the rhizobial proteins aligned, have a similar cysteine pattern as the one in the FLP, FlpA, and FlpB proteins that could suggest a regulatory mechanism involving sulfur bridge formation (Green et al., 2001).

The dimerization interface of *E. coli* FNR, from residues 140 to 159, is mainly composed of hydrophobic or aliphatic

aminoacids. Arginine residues in positions 140 and 145 together with the aspartate 154 regulate FNR activity in a positive and a negative way, respectively. From the FNR-like regulators of *R. etli*, only StoRd conserve the Arg140. Hydrophobicity is retained at positions 151 and 158 in all FNR-like proteins of *R. etli.* The hydrophobic character of residues that aligned with the proposed dimerization region of FNR from *E. coli* suggested that there might be a common interface in *R. etli* FNR homologs. Important differences between FNR-like proteins from *R. etli* and FNR from *E. coli* and more related homologs such as CydR from *Azotobacter vinelandi* might be the lack of Ser24 and Asp154 that are important for the dimerization regulatory mechanism (Jervis et al., 2009).

Aminoacids in the ARs of *E. coli* FNR are not highly conserved in the Fnr-like proteins of *R. etli*; nonetheless, some conserved aminoacids are present in more than one of the regulators.

The most conserved region of the FNR-like proteins is the DNA recognition motif: EXXSR. Glu209 and Arg213 of *E. coli* FNR make direct contact with the G–C and A–T pairs of the TTGAT sequence in the anaerobox region. Ser212 is responsible for the direct contact with the first T–A pair in the anaerobox consensus, in contrast to residues 209 and 213 this aminoacid is absent in the CRP–DNA recognition helix and provides a discriminatory contact for FNR together with Arg180 that is absent in FNR (reviewed by Green et al. (2001)). The DNA-binding motif of FNR from *E. coli*, ETVSR, is conserved in *R. etli* FnrN and FixKf proteins. StoRd and StoRf have a slightly different motif, EALSR, and in *R. etli* NnrR, as well as in their more closely related homologs, the first residue in the motif is a histidine instead of a glutamate residue (HTVSR). The serine and the arginine residues of the motif are conserved in all FNR-like proteins. Because Fnr-like proteins conserve the residues of the protein-DNA recognition helix (EXXSR) of FNR-like proteins, one could speculate that they can bind the Fnr-recognition site; however, the presence of these conserve residues is not always an indication of the ability of a protein to bind the anaerobox. An interesting case is that of FLP from *Lactobacillus casei.* Even though the recognition motif is conserved, this protein recognizes a different consensus, similar to but still different from that of FNR and CRP. It has been proposed that the presence of a proline instead of valine or arginine immediately before the glutamate of the recognition motif in FLP might be responsible for a change in the relative position of the recognition helix (Gostick et al., 1998). Interestingly StoR and SinR proteins have a proline before the first glutamate of the recognition motif. Whether they bind different consensus sequences and whether the proline has any role in its recognition still has to be demonstrated. FnrNd, FnrNch, and FixKf proteins have an isoleucine residue before the glutamate in the motif while NnrR has a leucine residue.

It is clear that even in the DNA recognition helix, the most conserved region of these proteins, there are some differences that might explain, at least to some extent, experimental observations regarding target specificity among the FNR-related proteins of *R. etli.*

Important questions are still pending for an answer: How different are the oxygen-sensing mechanisms of FnrN regulators in comparison with FNR from *E. coli*? Are all the Fnr-related regulators in this bacterium contacting the same subunits of the RNA polymerase? Are the cysteines present in the predicted dimerization interphase of StoRd and StoRf regulators playing any role in controlling oligomerization of these proteins? How does the presence of a Histidine instead of a Glutamate residue in NnrR-binding motif affect affinity or sequence discrimination? Does the Proline localized beside the binding motif of the StoRd and StoRf regulators have anything to do with the role of these proteins as negative regulators? These and many other questions could be formulated, and the ability to answer them will be instrumental for our understanding of the regulatory networks that the FNR-related regulators are involved in.

15.6 PREDICTION OF DNA-BINDING SPECIFICITY USING THREE-DIMENSIONAL MODELS OF FNR-RELATED PROTEINS OF *R. etli*

FixKf, FnrNch, and FnrNd share a highly conserved DNA-binding domain, whereas StoRd, StoRf, and NnrR regulators have notable differences in the conserved EXXSR motif. Both StoR regulators have a proline residue instead of a valine, lysine, or proline residue before the conserved glutamate in the binding motif. NnrR regulator has a histidine residue instead of the conserved glutamate. Furthermore, all the regulatory targets of NnrR known to date in *R. etli* have anaeroboxes with the consensus TTG-N8-CAA, but none of them have the TTGAT-N4-ATCAA consensus found in most of the *fix* genes. We hypothesized that the differences in the conserved DNA-binding motif can account for variations in binding affinity or target discrimination.

Recently, the crystal structure of $FixK_2$ of *B. japonicum* bound to a TTGAT-N4-ATCAA anaerobox was reported (Bonnet et al., 2013). Using the coordinates of this crystal [PDB 4I2O], we generated dimeric three-dimensional models of *R. etli* FixKf, FnrNch, StoRd, and NnrR using the software TFmodeller (Contreras-Moreira et al., 2007). The 3D footprint database is a useful tool recently developed for analyzing protein–DNA interfaces of crystal structures and three-dimensional models (Contreras-Moreira, 2010). Using the interactive footprint software of this database, we were able to analyze the protein–DNA contacts. The protein–DNA interfaces of the FixKf and FnrNch models are the same as in the $FixK_2$-anaerobox crystal structure.

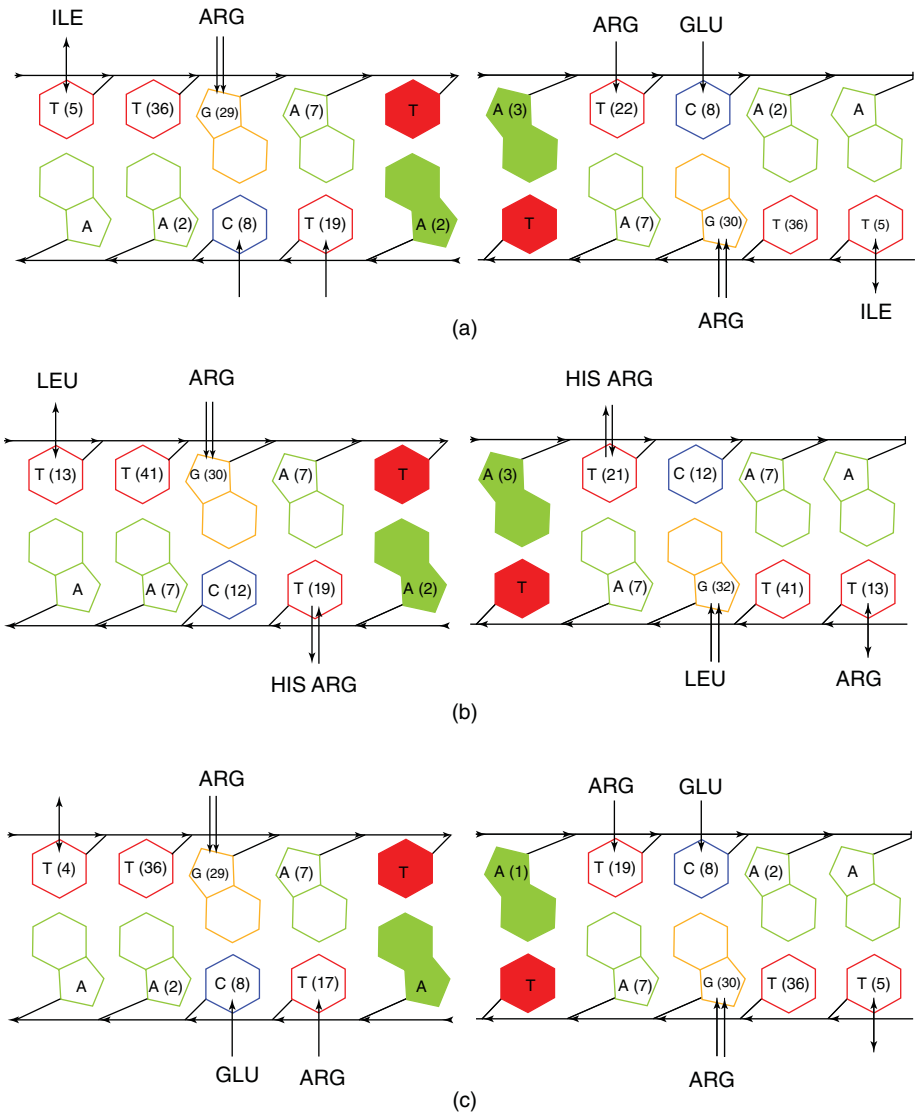

Figure 15.2 Protein–DNA interface of (a) FixKf, (b) NnrR, and (c) StoRd 3D models. Aminoacids of the conserved "VEXXSR" motif that contact the DNA are shown. Single-headed arrows symbolize a hydrogen bond formation, while double-headed arrows represent hydrophobic interactions. Full-colored nitrogen bases indicate predicted indirect contact with deformed DNA geometry. Numbers within the parenthesis inside the nucleotide bases indicate the number of side chain contacts.

Differences can be appreciated between the interface of the NnrR and StoRd model when compared to FixKf (Fig. 15.2). The presence of a histidine residue instead of a glutamate in NnrR results in the loss of a hydrogen bond with the 12th cytosine in the anaerobox (Fig. 15.2) and a new hydrophobic interaction is formed with the 11th thymine. The number of side chain contacts with the nitrogen bases is in general higher in the NnrR model when compared to FixKf (Fig. 15.2). In the case of StoRd, the only notable difference when compared with FixKf is that the presence of a proline instead of an isoleucine just before the conserved-binding motif is responsible for the loss of a hydrophobic contact with the first thymine of the anaerobox.

The algorithm of this footprinting software allows predicting the binding specificity of the regulators by the use of two types of position weight matrices (PWMs): contact and readout. The first one is calculated based on the number of contacts between side chains and nitrogen bases, while the readout PWMs are derived considering the array of scored atomic interactions at the interface and the set of sequence-dependent deformations inferred from the DNA coordinates (Contreras-Moreira, 2010). The predicted binding specificity of FixKf and StoRd is illustrated graphically by a consensus LOGO in Figure 15.3. From the *in silico* examination of the interfaces of FNR-like regulators in *R. etli*, one can suggest that specific changes in the binding motif, for instance the histidine and the proline in NnrR and StoRd, might be responsible for selective discrimination of specific targets. From the models it is also interesting to note that a hydrogen bond is formed between the arginine of the motif and the 11th thymine (5′ to 3′) of both strands of the anaerobox. In *norC*, for example, these nucleotides are substituted for guanine and adenine in each strand. Hydrogen bond formation does not occur when these

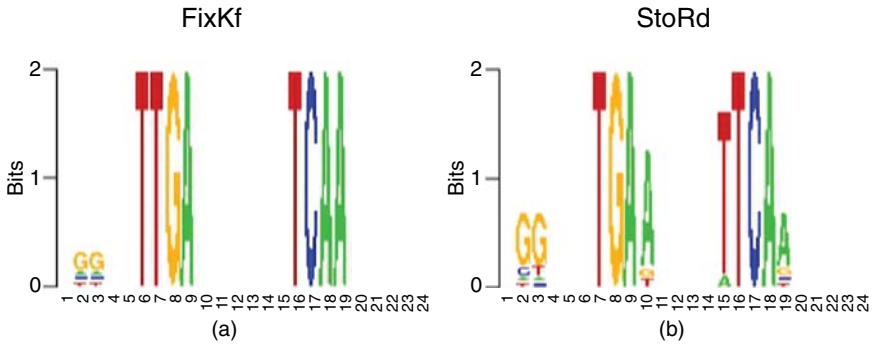

Figure 15.3 Binding specificity of FixKf (a) and StoRd (b) three-dimensional models. The LOGO represents the consensus predicted by the average of two types of position weight matrixes described in the text. It was generated by the web server 3D footprint using the "footprint your structure" software tool.

changes are incorporated *in silico*, although several side chain interactions with these nitrogen bases can be observed (data not shown). The absence of a thymine in position 11 allows the formation of a hydrophobic contact between the histidine in the motif of NnrR and the 13th position of the anaerobox. This interaction only occurs in the NnrR model (data not shown). Genetic and biochemical approaches will be necessary to analyze in deeper detail how modifications within the anaerobox sequence can affect binding affinity or shift sequence recognition by different FNR-like regulators.

15.7 PREDICTION OF FNR-RELATED TARGETS IN THE GENOME OF *R. etli* CFN42

FNR homologs of *R. etli* control the expression of genes that are important for respiration inside the nodule (*fixNOQP* and *fixGHIS* operons) and to alleviate nitrosative stress (*norCBQD*, *nirKVnnrRU* operons, *azuPf*, and *azuPd* genes). All these genes are expressed under microaerobic conditions both in culture and in symbiosis with legume plants. They have a conserved anaerobox with the consensus TTG-N_8-CAA. Furthermore, the presence of more than one anaerobox within the regulatory region of *nirK* allows fine-tuning of the transcription of this gene.

FNR from *E. coli* is known for being a global regulator that controls expression of several genes under anaerobic conditions. Recently, the chromosomal distribution of the targets of the regulator during exponential anaerobic growth was analyzed, and this study revealed 63 copies (Grainger et al., 2007). The presence of several FNR homologs in *R. etli* and the fact that variations in the DNA-binding domain may allow for sequence-affinity or sequence-discrimination differences make us speculate if different anaeroboxes or anaerobox-like sequences apart from the ones that have been previously described could be recognized by these regulators. A PWM was generated using the anaerobox sequence of the known targets of regulation of the six FNR-related proteins of *R. etli*. This matrix was used to scan the upstream region [−200 +1] of all genes of *R. etli* CFN42 using the matrix scan algorithm of the web server RSAT (http://rsat.ulb.ac.be/). This strategy revealed the presence of 303 putative anaeroboxes in the *R. etli* CFN42 genome. In some cases, two contiguous divergent genes shared one of these putative anaeroboxes. Anaeroboxes with the highest weight had the consensus TTG-N_8-CAA sequence. Other anaerobox-like sequences that lacked the consensus were predicted; however, until now there is no evidence that the FNR regulators can bind to them. The transcriptional profile under microaerobic conditions of a *R. etli* triple mutant in *fixL*, *fnrNd*, and *fnrNch* that no longer expresses the FNR-related proteins was compared to the wild-type strain. Apart from the known targets of regulation of these proteins, differences in the transcription of several genes were observed. Some of these genes have predicted anaeroboxes. Genes RHE_PD00022, *virB3a* (type IV secretion system protein), and $adhA_2$ (alcohol dehydrogenase) were downregulated in the absence of FNR regulators. These genes have anaeroboxes in their regulatory regions that highly resemble the anaerobox of known targets. Also genes with a less conserved anaerobox, lacking the consensus sequence TTG-N_8-CAA, were differentially expressed in the absence of FNR regulators. In this regard, it would be interesting to determine if these changes in expression are due to direct regulation and if these anaerobox-like motifs are important. Several genes with a highly conserved anaerobox were not clearly regulated by the FNR regulators under the conditions tested. It might be possible that the expression of some of these genes, although dependent at least on some level of the FNR regulators, have specific requirements that were not met in our experimental conditions. Interestingly, several highly conserved anaeroboxes (weight above five and with the consensus TTG-N_8-CAA) are located in plasmids pRet42d and pRet42f (16 and 18 unique sequences, respectively). The widespread transcription activity of the *R. etli* pRet42d observed under microaerobiosis (Girard et al., 1996) and the presence of several of the known targets of the FNR regulators on plasmid pRet42f suggest us that the occurrence of FNR regulators in these plasmids allowed the selection of adaptive *cis* mutations that favored survival

or increased fitness under microaerobic conditions either in free living or symbiotic bacteria. Same rational could apply for *fnrNch* in the chromosome of *R. etli*. Ulterior acquisition or recruitment of the megaplasmids brought robustness to the microaerobic response of this bacterium.

Some genes with unknown function were differentially regulated in the absence of the FNR proteins. A particularly interesting case is gene RHE_PD00022, transcription of this gene is drastically reduced in the absence of FNR regulators. The absence of either the FixL–FxkR two-component system or the FixKf regulator almost abolishes the expression of this gene. RHE_PD00022 gene has a canonical anaerobox with the consensus sequence TTGAT-N_4-ATCAA and is expressed at high levels both in culture at low-oxygen concentrations and in symbiosis. The predicted protein is annotated as a hypothetical protein and possesses three tandemly repeated BON (Bacterial OsmY and nodulation) domains. This type of domain is mostly unexplored and little is known about its function (Yeats and Bateman, 2003). The most well-characterized protein baring BON domains is OsmY, a protein that confers protection to osmotic stress in *E. coli* (Yim and Villarejo, 1992). The aminoacids conserved in the BON domain are suggestive of a binding function. It has been hypothesized that the BON domains of OsmY help to avoid shrinkage of the cell by contacting the phospholipid interphase within the periplasmic space (Oh et al., 2000; Liechty et al., 2000). Another BON-domain baring protein is NolX, a protein involved in the secretion of a pilus structure that determines host specificity in rhizobial nodule formation. The phyletic distribution of these proteins suggests that most Proteobacteria have one or two BON-domain baring proteins. These proteins are more extended in the *Burkholderia* genus. Unusually three repeated BON-domain architectures as the one in RHE_PD00022 are only present within *Burkholderia* proteins and proteins encoded in the symbiotic or pathogenicity plasmids of *Ralstonia* and *Rhizobiales*. This distribution strongly suggests a role for these proteins in cell–cell interaction or host invasion (Yeats and Bateman, 2003). Interestingly, gene RHE_PC00107 in pRet42c is predicted to have a CBS and a BON domain, these two domains are frequently found together. It also has a highly conserved anaerobox, and even though it did not appear to be differentially regulated in our expression analysis its neighbor $adhA_2$ (RHE_PC00106), which also has a highly conserved anaerobox, was dependent on FNR regulators. Gene RHE_PF00490 also has a CBS domain but lacks a BON domain. This gene also possesses a highly conserved anaerobox. These genes are encoded in plasmids that are crucial for symbiosis and nitrogen fixation; it will be of great relevance to analyze if they play a role in these crucial processes of rhizobial physiology.

15.8 CONCLUDING REMARKS

FNR-like regulators are crucial for nitrogen fixation in Rhizobia. FixK and/or FnrN are responsible for the activation of the *fix* genes in *S. meliloti*, *B. japonicum*, *R. leguminosarum*, and *R. etli*. However, in most of these bacteria, there is more than one regulator of this type and their function has not been clarified. From the study of the regulation of *fix* genes in *R. etli*, we have learned that several regulators of the same family can be used to shape an adapted regulatory circuit. The inclusion of regulators with similar specificities such as in the case of FixKf, FnrNch, and FnrNd can make a regulatory network more robust. However the ability of FnrN regulators to directly detect oxygen in addition might allow fine-tuning of the response by modifying the detection threshold of the oxygen signal. The responses to signals that are often encountered together in a particular environment probably evolve to include regulators that recognize very similar DNA targets although with some differences in specificity or affinity. It is well known that low oxygen and nitrogen oxide are signals that dwell within the nodules. It is tempting to speculate that a relatively small number of changes were required to modify the specificity for preexisting anaeroboxes that were recognized by a low-oxygen responsive regulator to now be targeted by a protein with a highly similar DNA-binding motif that has acquired the novel function of nitrogen oxide sensor. Having two different sensors for separate signals allows setting up a hierarchical response in a stepwise manner. NnrR and FixKf seem to fit in that kind of hierarchical response in *R. etli*.

StoRd and StoRf play a role as modulators within the regulatory cascade that control the expression of *fix* genes in *R. etli*; however, until know it has not been shown that these regulators can bind directly to the anaerobox. The identification of the binding site of these regulators in the regulatory region of their reported targets will shed light on the molecular mechanisms that promote positive and negative regulation at low oxygen levels in *R. etli*.

Several anaeroboxes have been predicted in the regulatory regions of the genes encoded in the genome of *R. etli*; however, only few efforts have been made so far to determine the functionality of these sequences. With the improvement and the development of novel genomics approaches, it has become an obligation to pursue a deeper but broader understanding of the role of FNR-like regulators in rhizobial physiology. The inclusion of several members of the FNR/CRP superfamily in the *fix* regulatory cascade of *R. etli* makes this regulatory model attractive for the study of the impact of cross talk and the mechanisms that have been selected to avoid its counterproductive effects.

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Section 4

Taxonomy and Evolution of Nitrogen Fixing Organisms

Chapter 16

Exploring Alternative Paths for the Evolution of Biological Nitrogen Fixation

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16.1 INTRODUCTION

Biological nitrogen fixation is curiously restricted to prokaryotes and is associated with a diversity of microorganisms that represent a wide range of physiologies (Boyd et al., 2011a, 2011b; Dos Santos et al., 2012). Since nitrogenase is very sensitive to oxygen, different classes of organisms have evolved different mechanisms to catalyze nitrogen fixation in an oxic environment (Gallon, 1992; Berman-Frank et al., 2003). Probably the most recognized mechanism is associated with symbiotic nitrogen fixation where plants provide a microaerobic niche by producing leghemoglobin that sequesters oxygen in nodules, thereby maintaining very low oxygen tensions (Ott et al., 2005). Nitrogen fixation occurs under anoxic conditions in strict or facultative anaerobes and nonfilamentous cyanobacteria or in the heterocysts of filamentous cyanobacteria. In obligate aerobes, the nitrogen-fixation apparatus is protected by what has been described as respiratory protection whereby high rates of respiration ensure the consumption of oxygen at the cell membrane, thereby maintaining an oxygen-free cytoplasm. It is likely that these mechanisms emerged later in the evolutionary history of biological nitrogen fixation owing to the increased complexity of *nif* gene clusters in microorganisms adapted to fixing nitrogen in air (Boyd et al., 2011a). The simplest assemblages of specific genes associated with nitrogen fixation occur in strict anaerobes, but tracing the evolutionary trajectory and identifying the most ancient nitrogen fixers present in extant biology has been a challenge.

16.2 HOW ANCIENT IS BIOLOGICAL NITROGEN FIXATION?

Many would like to place biological nitrogen fixation as an ancient and perhaps even primordial process. This is due to the essential requirement of fixed nitrogen for life and the presumed limiting amount of fixed nitrogen in the early reducing Earth when the polyoxyanions of nitrogen (nitrate and nitrite) would likely be present in vanishing low quantities if at all (Falkowski, 1997; Fani et al., 2000). Apart from models based on atmospheric chemistry (Yung and McElroy, 1979; Kasting and Walker, 1981; Navarro-González et al., 2001), little is known, however, about the availability of ammonia or other reduced forms of nitrogen over the course of geological time. One can however ask the general question of whether the overall occurrence of the biochemical apparatus present in extant biology, nitrogenase, and its associated functionalities are consistent with a primordial origin by focusing the question of whether nitrogenase was likely to be a property of a Last Universal Common Ancestor (LUCA). Biological nitrogen fixation is not found associated with any eukaryote, but the process is widely distributed among bacteria with a very limited distribution among methanogenic archaea. Although widely distributed both

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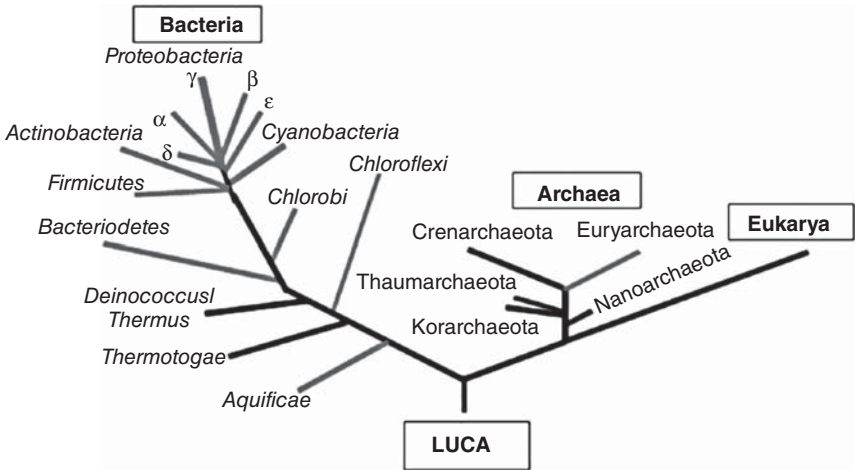

Figure 16.1 A taxonomic tree indicating lineages where representative organisms have been identified that harbor *nif* (overlaid in gray).

taxonomically (Boyd et al., 2011a) and ecologically (Zehr et al., 2003), the process is far less than universal and unlike processes and functionalities that we ascribe to properties of LUCA, the process is not commonly associated with deeply rooted lineages identified by 16S ribosomal DNA evolutionary trajectories (Fig. 16.1).

16.3 WHAT ARE THE MOST DEEPLY ROOTED ORGANISMS THAT HARBOR NITROGENASE AND PRESUMABLY FIX NITROGEN?

To answer this question, one must define a set of criteria to establish what genes are required for nitrogen fixation today, and hypothetically which were required to fix nitrogen in ancestral populations. This is not as simple as in many other systems where the presence of one or more specific gene products that encode the enzyme provides the basis for an active function. This is due to the requirement of additional gene products that are involved in the biosynthesis of the unique cofactors (Rubio and Ludden, 2005, 2008; see Chapter 7). From previous genomic, biochemical, and molecular genetic studies on biological nitrogen fixation from different microbial sources, we and others have established a criterion defining the minimal set of genes required to synthesize an active nitrogenase (Boyd et al., 2011a; Dos Santos et al., 2012). This criterion stipulates that the structural genes *nifH*, *nifD*, and *nifK* and three additional FeMo-cofactor biosynthetic genes *nifE*, *nifN*, and *nifB* must be present in an operon for it to be considered to encode for a functional nitrogenase. The criteria is based mainly on the observation that deletion mutation analysis of *nifE* and *nifN* (Ugalde et al., 1984; Jacobson et al., 1989; Roll et al., 1995; Hu et al., 2005) and *nifB* (Shah et al., 1994; Christiansen et al., 1998) results in the production of an inactive FeMo-cofactor-less nitrogenase. Moreover, the *nif* clusters of all sequenced nitrogen fixers that have been characterized minimally have these gene products (Boyd et al., 2011a). Using this criterion, we identified all putative diazotrophs with sequenced genomes and exploited specific genetic events (e.g., gene fusions, duplications) that were involved in the evolution of the biosynthetic pathways to identify which extant organisms harbor the oldest nitrogenase.

The first relationship that was exploited was the relationship between the structural gene products that encode the MoFe protein, NifDK, and the paralogous NifEN complex, which functions as a scaffold for FeMo-cofactor biosynthesis. Primary amino acid sequence comparisons of NifD, NifK, NifE, and NifN reveal significant homology, indicating that these gene products have evolved from a common ancestor. It has been suggested that two sets of gene duplications resulted in these four related gene products. The first duplication of *nifD*, which is presumed to be ancestral for reasons discussed later, was duplicated to *nifK* generating the heterotetrameric MoFe protein (Fani et al., 2000). Subsequently, *nifD* and *nifK* were duplicated in tandem to form *nifEN*, which encodes for a heterotetrameric complex that functions as a dedicated scaffold for FeMo-cofactor biosynthesis (see Chapter 7). Phylogenetic reconstruction of paralogous NifD, NifK, NifE, and NifN proteins reveals distinct clades that correspond with each protein, with NifE and NifN proteins nested among NifD and NifK proteins, respectively, consistent with this evolutionary trajectory (Boyd et al., 2011a, 2011b). With regard to the overarching question of the identity of the most ancient nitrogen fixers among sequenced extant organisms, the NifD and NifK proteins associated with hydrogenotrophic methanogens form basal branches (Fig. 16.2), suggesting that nitrogenase in these taxa is most reminiscent of the ancestor. Such results strongly suggest an origin for nitrogen fixation in an anoxic environment, which is consistent with the oxygen sensitivity of this enzyme complex, and our suggestion that the mechanisms organisms have adapted

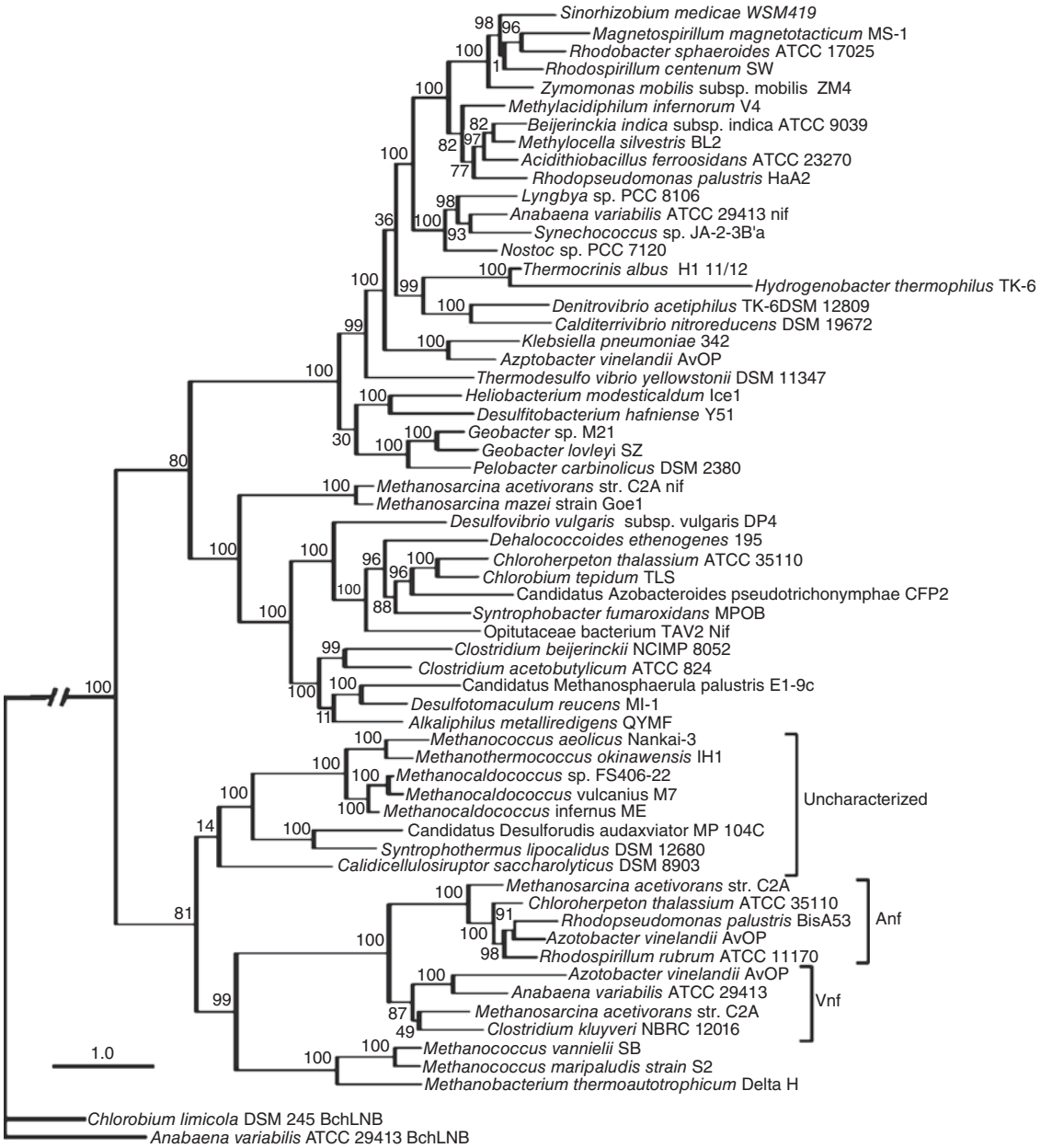

Figure 16.2 Maximum-likelihood phylogenetic reconstruction of a concatenation of Nif/Anf/Vnf and uncharacterized HDK proteins. Individual proteins were aligned, concatenated, and subjected to evolutionary reconstruction as described previously (Boyd et al., 2011b). The composition of the active-site clusters other than those that correspond to Nif (e.g., Vnf, Anf, uncharacterized) is overlaid on the phylogeny. Bootstrap values are indicated at the nodes.

to fix nitrogen in the presence of oxygen are more recent innovations.

Reinforcement of these results which implicate hydrogenotrophic methanogens as the oldest nitrogen-fixing organisms, the evolution of the essential NifB protein offers additional support. NifB is a radical generating *S*-adenosylmethionine (SAM)-dependent enzyme that is involved in generating the hexacoordinated carbide at the center of the FeMo-cofactor (Wiig et al., 2012), which is the presumed key structural determinant of nitrogenase function (see Chapter 7). Most NifB proteins of extant organisms exist as putative two-domain protein composed of a radical SAM functionality and a carrier functionality that is the result of a fusion between the core NifB protein and a carrier protein that still exists as a standalone functionality termed NifX (Rubio and Ludden, 2008). Methanogens lack the associated NifX domain as a component of NifB and as such would have emerged prior to the fusion of these two functionalities in the evolution of NifB (Boyd et al., 2011a).

16.4 ARE ALTERNATIVE NITROGENASES EVOLUTIONARY ANCESTORS OF Mo-NITROGENASE?

The evolutionary trajectory of the different metal-containing nitrogenases has been an interesting question since their discovery nearly 30 years ago now. It has been suggested that nitrogen fixation by alternative nitrogenases might have preceded Mo nitrogenase in the early Earth and were the dominant nitrogenases prior to the *Great Oxidation Event* and the advent of oxygenic photosynthesis (Anbar and Knoll, 2002; Raymond et al., 2004). This suggestion was largely based on the presumed limited availability of Mo under the reducing environment of the early Earth (Anbar et al., 2007; Anbar, 2008) where most Mo would have been complexed in insoluble Mo-sulfides (Helz et al., 1996). Although this logic is sound and the potential for alternative nitrogenases as ancestors of Mo-nitrogenase is a rational suggestion, there are a number of both direct and indirect observations that indicate that this is not likely to be the case.

The alternative nitrogenase functions with either V in the place of Mo (V-dependent nitrogenase) or in the absence of any heterometal (Fe-only nitrogenase). Biochemical studies indicate that alternative nitrogenases are less efficient owing primarily to the substitution of either V or Fe for Mo in the FeMo-cofactor such that V-nitrogenase and Fe-only nitrogenases function with either at FeV-cofactor or what has been termed an FeFe-cofactor, respectively (Eady, 1996). Alternative nitrogenases are found to occur only in a small number of organisms and, to date, have never been observed in genomes that do not also encode a Mo-nitrogenase (Raymond et al., 2004; Boyd et al., 2011a). Gene clusters encoding the structural components of the alternative nitrogenases only possess a fraction of the required cofactor biosynthetic genes required for FeMo-cofactor biosynthesis (Boyd et al., 2011b). Transcriptional analysis of the model nitrogen-fixing organism *Azotobacter vinelandii* indicating nitrogen fixation by alternative nitrogenases (Vnf or Anf dependent) involves the expression of a number of FeMo-cofactor synthetic gene products located in the *nif* operons and is also required for the expression of active Mo-nitrogenase (Wolfinger and Bishop, 1991; Hamilton et al., 2011). These observations make it difficult to rationalize an ancestry involving the features of an extant alternative nitrogenase without invoking fairly extensive gene loss and/or rearrangements.

Lacking key dedicated biosynthetic gene products (E, N, or B), alternative nitrogenase does not obey the criteria that were put forth above that define the ability to catalyze nitrogen fixation. Inclusion of Vnf and Anf structural genes (Anf/VnfD and K) in the internally rooted phylogenetic analysis of NifD, NifK, NifE, and NifN amino acid sequences reveal ambiguous results as to which form of nitrogenase is ancestral (Boyd et al., 2011a). Previous studies examining the evolutionary trajectories of NifD alone or concatenations of NifH and NifD also lead to ambiguous results (Raymond et al., 2004). Since all three nitrogenases (Mo-, V-, and Fe-only) have dedicated structural proteins (H, D, and K), we recently conducted a phylogenetic study in which alignments of all three protein sequences were concatenated (Boyd et al., 2011b). In this study, the phylogenetic relationships were better resolved and a clear pattern was revealed (Fig. 16.2). Well-supported phylogenetic trees indicate that the alternative nitrogenases are nested among basal branching Mo-nitrogenase representatives. The phylogenetic and bioinformatics studies, as are all of the studies described in the chapter, are limited by only being able to analyze sequenced extant organisms. However, the results are compelling and consistent with biochemical and physiological data that together provide a strong case for reevaluating the dogma associated with basing the ancestry of nitrogenase solely on the availability on Mo in the geologic record.

16.5 WHAT IS THE NATURE OF THE METAL COMPLEMENT OF "UNCHARACTERIZED NITROGENASES"?

The assignment of the metal cofactor at the active site of nitrogenase homologs as described in the aforementioned phylogenetic studies is based on patterns of clustering with representative homologs that have been biochemically characterized to some extent. There are, however, a number of deeply rooted lineages that do not contain characterized nitrogenase proteins (Mehta and Baross, 2006; Boyd et al., 2011b; Dos Santos et al., 2012) (Fig. 16.2). Some of these uncharacterized nitrogenases do not obey our established criteria and lack homologs of the biosynthetic gene, *nifN*, required to be considered as a *bona fide* nitrogenase.

As mentioned previously, alterative nitrogenases are less efficient as nitrogen reduction catalysts, and previous biochemical studies have also shown interesting differences in the reduction of other substrates, including hydrogen, acetylene, and carbon monoxide. Alternative nitrogenases produce a larger proportion of hydrogen as a product in the nitrogenase reaction than does Mo-nitrogenase (Eady, 1996). Acetylene reduction catalyzed by the Mo-nitrogenase results in ethylene as the sole product in contrast to the alternative nitrogenases that produce detectable quantities of ethane in addition to ethylene. The recent observation that nitrogenases are capable of hydrocarbon production with carbon monoxide as a substrate (Lee et al., 2010; Hu et al., 2011) indicates that Mo-nitrogenases and V-nitrogenases have differing catalytic efficiencies for hydrocarbon production with V-nitrogenase having higher catalytic rates. Interestingly, it has been shown that simple site-specific

amino acid substitutions of Mo-dependent nitrogenase can affect increased hydrocarbon production on the order of that observed for that of the V-dependent nitrogenase (Yang et al., 2012). These results indicate that the combination of the metal content and cofactor protein environment that make up the structural determinants account for the subtle differences in substrate reduction properties of the different metal-dependent nitrogenases. Recently, we conducted a fairly exhaustive study of the polypeptide environment of the deeply rooted uncharacterized nitrogenase based on homology models (McGlynn et al., 2012). This work clearly indicated that the uncharacterized nitrogenases were more likely to be Mo-dependent nitrogenases than alternative nitrogenases. Given the organisms that possess uncharacterized nitrogenases occupying anaerobic niches, the results of this work is in line with our previous studies indicating that the oldest extant nitrogen-fixing organisms are anaerobes and that biological nitrogen fixation had its origins in an anoxic environment (Boyd et al., 2011a, 2011b). In addition, the observation that these deeply rooted uncharacterized nitrogenases are likely to be Mo-dependent further supports our previous observations indicating that Mo-dependent nitrogenases are ancestral to the alternative nitrogenases.

16.6 IS THERE AN EVOLUTIONARY RELEVANCE TO NITROGENASE PROMISCUITY?

The ability of nitrogenases to reduce other substrates such as acetylene and cyanide and their ability to convert carbon monoxide to hydrocarbon products has enticed some to propose that nitrogenase may have its evolutionary roots in one or more of these catalytic activities (Fani et al., 2000; Hu et al., 2011). This is an interesting idea, given that the barrier for these reactions is lower and this might afford a somewhat stepwise path to achieving an enzyme capable of overcoming the enormous activation barrier of dinitrogen activation (Rees, 1993). Nitrogenase enzymes with the dedicated physiological function of reducing these types of substrates or analogous compounds have not been identified in extant biology, and it is difficult to envision conditions that would represent a strong selective pressure to evolve such enzymes. Recent work involving the observation that Mo- and V-dependent nitrogenases are capable of hydrocarbon production from carbon monoxide has been used to suggest a link between nitrogenase-dependent production of reduced carbon and nitrogen (Lee et al., 2010; Hu et al., 2011). Imagining a selective pressure that would lead to this complicated and presumably ATP-dependent mechanism for generating reduced carbon in early life is difficult, especially in light of the presence of other viable mechanisms present in extant biology. The Wood–Ljungdhal pathway is a prime example and the key enzyme, the carbon monoxide dehydrogenase/acetyl CoA synthase (Ragsdale, 1991), for example, has a much more common occurrence in deeply rooted microorganisms (Techtmann et al., 2012) than nitrogenase and is by all accounts a more ancient enzyme. We presume that nitrogenase evolved in response to selective pressure of fixed nitrogen availability, and nitrogenase promiscuity and the ability to reduce other substrates is a product of the evolution of a redox enzyme that can overcome the largest activation barrier in biology.

16.7 WHAT IS THE EVOLUTIONARY ORIGIN OF NITROGENASE?

The evolutionary origin of nitrogenase is a very interesting question and is framed by a number of traditional paradigms that are not strongly tied to the observations we can glean from extant biology. In today's world, the availability of fixed nitrogen limits global nutrition and so there is a natural desire to place the emergence of biological nitrogen fixation as a very early event and perhaps even a primordial process. However, even the simplest evolutionary observations such as the aforementioned limited distribution of biological nitrogen fixation in deeply rooted lineages is not consistent with biological nitrogen fixation being a property of LUCA. The history of nitrogen availability is not something that can, as of yet, be ascertained from the geologic record, so the time when the selective pressure was sufficient to affect the emergence of such a complicated biochemical process is unclear. Some insights however can be assembled from other evolutionary relationships including the closely related protein complexes associated with chlorophyll and bacteriochlorophyll biosynthesis and the related enzyme complex putatively involved in F430 biosynthesis found most commonly in methanogens (Raymond et al., 2004; Boyd et al., 2011b) (Fig. 16.3).

There is an emerging body of work on the biochemistry of the dark operative protochlorophyllide reductase complex involved in bacteriochlorophyll biosynthesis (Fujita and Bauer, 2000; Brocker et al., 2008, 2010; Sarma et al., 2008; Muraki et al., 2010; Moser et al., 2013). In brief, the enzyme catalyzes the stereo-specific reduction of the C17–C18 double bond of the D-ring of protochlorophyllide to form a chlorophyllide. Presumably, the nature of this stereo-specific reduction is facilitated by an analogous gated electron transfer mechanism required in biological nitrogen fixation and thus involves an analogous enzyme complex, which in bacteriochlorphyll biosynthesis involves an Fe-protein analogous component, BchL, and a MoFe protein analogous component, BchNB. At the site of active site cofactor binding in nitrogenase, BchNB possesses a

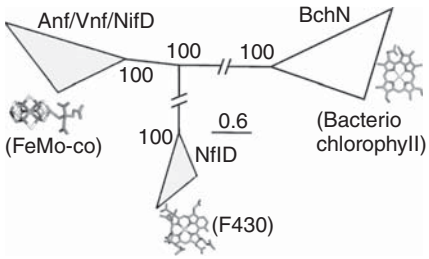

Figure 16.3 Phylogenetic relationships between Anf/Vnf/NifD, BchN, and NflD proteins indicate a clear evolutionary ancestor (Boyd et al., 2011b). Parsimony would suggest that the ancestor is similar to the NflD or BchN protein. The ancestor of these protein complexes (at the trifurcation point of the tree) likely encoded a single structural protein approximating NflD. An ancient duplication followed by independent evolution of the second NflD copy yielded the precursor to the heterotetrameric BchNB and NifDK complex. See Figure 16.4 for a schematic describing this evolutionary trajectory.

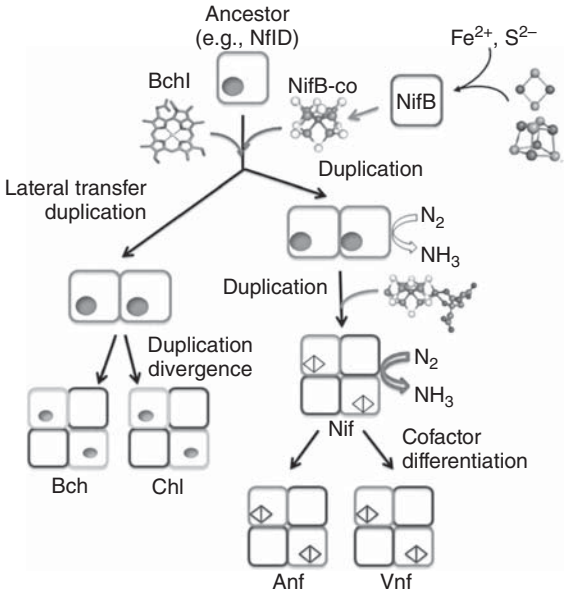

Figure 16.4 Hypothetical scheme depicting the evolution of nitrogenase from its protein ancestor. NifB, which catalyzes the formation of the FeMo-co precursor NifB-co from simple FeS clusters and/or ferrous iron/sulfide is presumed to have been paramount in providing the selective pressure to evolve a protonitrogenase. Here, NifB-co would have serendipitously nested in the cavity of the nitrogenase ancestor (e.g., BchNB- or NflD-like), which would have had the ability to reduce N_2, although without high efficiency. Duplication of nifDK and subsequent diversification resulting in nifEN yielded the ability to further mature NifB-co into FeMo-co.

cavity for protochlorophyllide binding, and substrate reduction involves BchL-dependent electron transfer reactions to affect substrate reduction.

From a structural perspective relating the arguably simpler protochlorophyllide reductase to the more complex cofactor-containing nitrogenase through parsimony would dictate invoking that the least common ancestor is the one with most simple components. That is to say, the simplest evolutionary trajectory is one in which the common ancestor approximates the structure of the protochlorophyllide reductase or a cofactor-less nitrogenase (Brocker et al., 2008; Muraki et al., 2010). The mechanism of cofactor biosynthesis is an additional source of insight when thinking of plausible scenarios for the evolution of nitrogenase (Rubio and Ludden, 2008; Hu and Ribbe, 2013). The final step in nitrogenase enzyme maturation is in fact the insertion of a preformed cofactor into a cofactor-less nitrogenase that for all intent and purposes approximates the salient structural features of the protochlorophyllide reductase (Schmid et al., 2002). Interestingly, neither the cofactor-less nitrogenase nor the cofactor on its own have nitrogen-reducing activity, observations that when considered in the context of evolution narrow down viable scenarios. We have spent considerable time attempting to envision evolutionary paths for nitrogenase in which a step-wise process could emerge such that structural determinants for catalysis derived from the cofactor and structural protein could be brought together to form an active nitrogen-reducing enzyme. When approached from this perspective, the most parsimonious scenario involves a common ancestor with an open cavity resembling protochlorophyllide reductase (Brocker et al., 2010; Muraki et al., 2010) (Fig. 16.4). This reductase serendipitously binds a modified iron–sulfur cluster fragment, and the result is a protonitrogenase with a selectable level of nitrogen-reducing activity. This could be in the form of a radical SAM-modified carbide iron–sulfur cluster similar to the cofactor intermediate proposed to form at an early state in FeMo-cofactor biosynthesis (Soboh et al., 2010; Wiig et al., 2012). In this scenario, additional selective pressure would exist to refine the process to the complicated biosynthetic pathway observed in extant biology.

ACKNOWLEDGMENTS

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Chapter 17

Phylogeny, Diversity, Geographical Distribution, and Host Range of Legume-Nodulating Betaproteobacteria: What Is the Role of Plant Taxonomy?

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17.1 INTRODUCTION

It has long been known that many species in the Leguminosae (Fabaceae) form a beneficial symbiotic interaction with N-fixing soil bacteria in the genus *Rhizobium*, as well as with other N-fixing bacteria in the Alphaproteobacteria class, such as *Bradyrhizobium*, *Azorhizobium*, *Ensifer* (*Sinorhizobium*), and *Mesorhizobium* (members of these genera are collectively termed "rhizobia"). This interaction involves the infection of legumes by the rhizobia and the development on their roots (and occasionally their stems) of structures termed nodules (see Chapter 4; Sections 9–12). The rhizobia proliferate within the developing nodules and establish an endosymbiosis within the plant cells, which allows the bacteria to fix N at a high rate, converting it to ammonia, which is then transferred to the host plant and used for its growth. Nodulated N-fixing symbioses have allowed legumes to colonize nutrient-poor soils, and many are pioneer species (Sprent, 2009). Nodulated legumes are considered to be the main contributors of biologically fixed N to pristine (i.e., unspoilt) ecosystems (Cleveland et al., 1999), and are thus essential to their ecology. Partly owing to its size and range of biomes, Brazil is one of the main centers of radiation of the Leguminosae in the World (Lewis et al., 2005), and it thus harbors an extraordinary diversity of legume species (including many endemics) that can

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nodulate with many different rhizobial species (Sprent, 2009). Examples of Brazilian biomes where legume–rhizobial symbioses proliferate and even dominate are parts of Amazonia, the Cerrado, the Caatinga, and the Mata Atlantica (Moreira et al., 1992; de Faria et al., 1984; Sprent et al., 1996; Bontemps et al., 2010; dos Reis et al., 2010; Freitas et al., 2010). In addition to their ecological importance, several nodulated legumes are used as crops for human and animal consumption.

In the last 10–12 years, there has been increased focus on a newly described group of bacteria that can nodulate legumes. These are not related to conventional "rhizobia" in the Alphaproteobacterial genus *Rhizobium*, but are all in the Betaproteobacteria class, which contains genera such as *Burkholderia*, and are thus sometimes termed "beta-rhizobia" (Gyaneshwar et al., 2011; see also Chapter 89). Originally, it was doubted that legumes could be nodulated by bacteria in other classes of the proteobacteria, such as the Betaproteobacteria, although N_2 fixation is common in these classes, the bacteria within them had long been considered to be exclusively free-living and/or loosely associated with plants, and not considered as nodulating symbionts (Gyaneshwar et al., 2011; see Chapter 88). Nodules, with their ample supply of nutrients and their enclosed protective environment, are an attractive niche for a wide variety of nonsymbiotic bacteria that have the capability to colonize plants opportunistically (Sprent 2009; Lima et al., 2009), but most examples of "nonrhizobial" isolates from nodules have failed to fulfill "Koch's postulates" (i.e., they cannot renodulate their hosts). However, shortly after the first claims of legume-nodulating "β-rhizobia" emerged in 2001 for *Ralstonia taiwanensis* (now renamed *Cupriavidus taiwanensis*) isolated from invasive *Mimosa* spp. in Taiwan (Chen et al., 2001) and for *Burkholderia* spp. isolated from legumes in South Africa and French Guiana (Moulin et al., 2001), conclusive evidence for nodule formation and effective nitrogen fixation ability was obtained (Chen et al., 2003a, 2005a, b; Elliott et al., 2007a, b; see also Chapter 89). In all of these studies, green fluorescent protein (GFP) marked strains of *C. taiwanensis* and various *Burkholderia* strains were used to perform microscopical analyses of the nodulation processes on various legumes, but particularly on Brazilian native and invasive *Mimosa* spp., and they thus demonstrated that (i) Betaproteobacteria could fix N_2 symbiotically within nodules on these legumes and (ii) that the infection and development of N_2-fixing nodules with Betaproteobacteria were essentially the same as that described for many other legumes by "normal" rhizobia (Sprent, 2009).

The early studies of beta-rhizobia suggested that they might have a close affinity with the large legume genus *Mimosa* (Mimosoideae), which contains more than 500 species, with at least 300 of them native or endemic to Brazil, with the Cerrado and Caatinga as their main centers of radiation (Simon et al., 2011). This affinity has been demonstrated by recent studies by Bontemps et al. (2010) and dos Reis et al. (2010), in which it was shown that 70 *Mimosa* spp. (many of them endemics to the Cerrado and Caatinga) were almost exclusively nodulated by *Burkholderia*, see also Chapter 89. Therefore, based on these studies (and others, as we shall detail later in this review), it is reasonable to suggest that Brazil is a major center of symbiotic *Burkholderia* diversity, but there is at least one other, centered in South Africa, in which several endemic papilionoid legumes (*Cyclopia*, *Lebeckia*, *Rhynchosia*) are nodulated by strains closely related to *B. tuberum* (Elliott et al., 2007b; Gyaneshwar et al., 2011) or belonging to new species (De Meyer et al., 2013a, b). In this context, it is interesting to note that even *Mimosa*-nodulating *Burkholderia* strains, such as *B. phymatum* STM815 can be found associated with papilionoid legumes, such as common bean (Talbi et al., 2010). Indeed, papilionoid-nodulating *Burkholderia* may be of potential agricultural importance in poor soils, especially those that have high-salinity levels, or suffer from extremes of pH (high or low) (Gyaneshwar et al., 2011).

The aim of this chapter is to update the recent review by Gyaneshwar et al. (2011), particularly with regard to the following topics:

1. Several new species of *Burkholderia* have been described, and we shall put them into context with regard to their legume hosts and their geographical distribution, and also to evaluate how they might (or might not) assist in current attempts to divide the genus *Burkholderia* into two new genera, separating the "pathogenic" from the "beneficial" species (see also Chapter 89).
2. It is now clear that *Cupriavidus* symbioses are not confined to *C. taiwanensis*, and in addition to soil factors (such as pH and the presence of heavy metals) there are possible geographical and plant phylogenetic factors underlying these symbioses.
3. Evaluation of potentially new "rhizobial" taxa within the Betaproteobacteria (see also Chapter 89).
4. Coevolution between beta-rhizobia and their legume hosts, with particular reference to the Mimosoideae.

17.2 BURKHOLDERIAS ARE HIGHLY DIVERSIFIED SYMBIONTS OF LEGUMES

The legume-nodulating burkholderias are closely related to plant-beneficial, often diazotrophic, endophytic, and environmental *Burkholderia* species and strains, but not to the phytopathogenic/pathogenic group of burkholderias (including the "*B. cepacia* complex," Fig. 17.1) (Suarez-Moreno

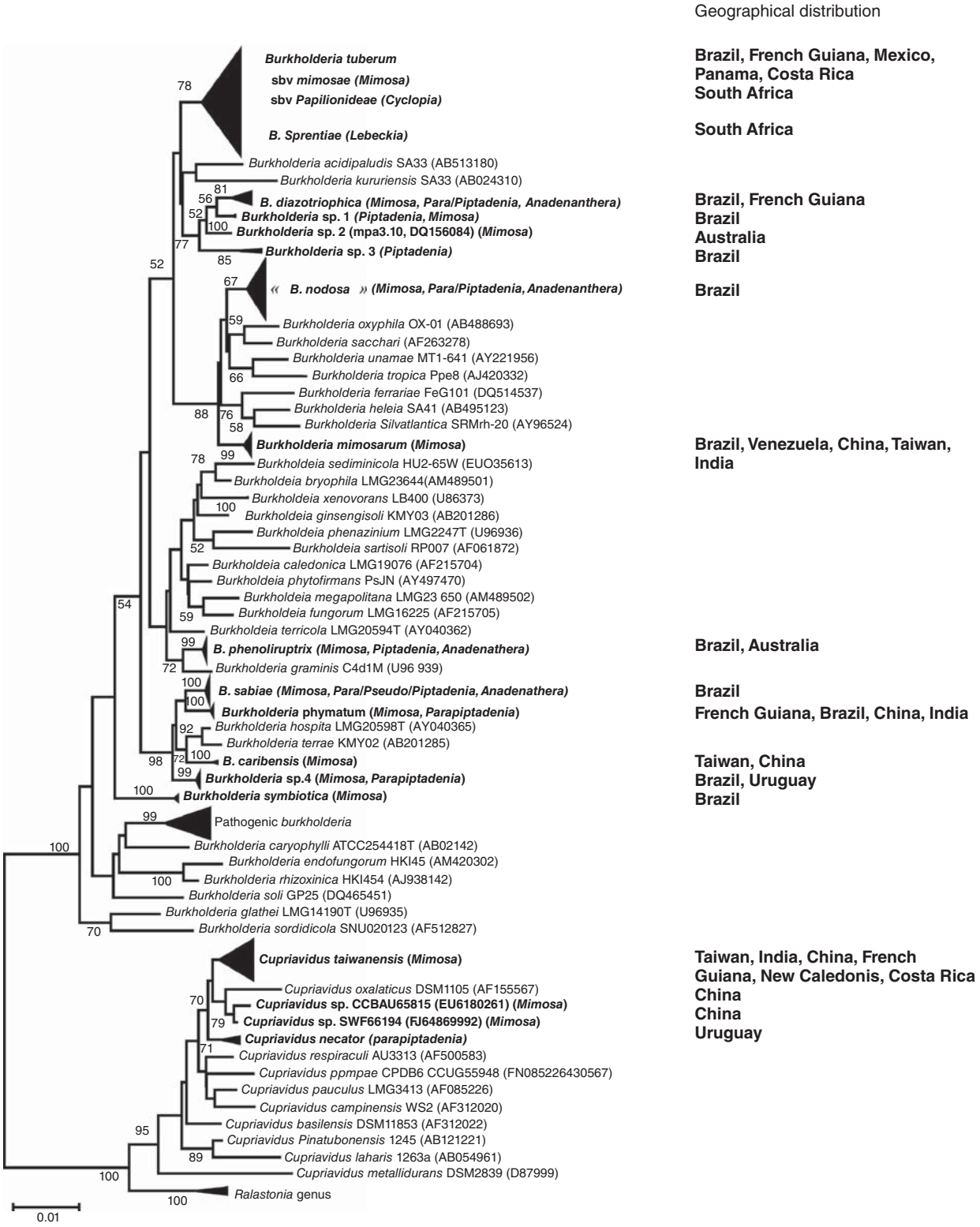

Figure 17.1 16S rRNA phylogeny of the *Burkholderia* genus. The tree was built using the neighbor-joining method, based on evolutionary distances computed using the maximum composite-likelihood method, with MEGA5 software. Bootstraps percent values (1000 replicates) are indicated at each tree node (if >50%). The analysis involved 299 nucleotide sequences, and 1273 positions in the final dataset. Some clades were summarized as triangles to simplify the figure. *Burkholderia* legume symbiont clades and species are indicated in bold. The legume host of origin of the isolates is indicated in parentheses, while their geographical origin is given at the right-hand side of the tree (see also Chapter 89).

et al., 2012). It is considered that the legume-nodulating burkholderias may have originated from the environmental/endophytic ones. A reasonable scenario is that approximately 50 mya, shortly after the first emergence of the legumes at 60 mya and coinciding with the evolution of their ability to nodulate (Doyle, 2011), the environmental/endophytic ancestors of the legume-nodulating burkholderias "added" *nod* genes (from an unknown donor) to their pre-existing *nif* genes (Bontemps et al., 2010). These potentially nodulating burkholderias then encountered several legume ancestors, became their symbionts, and then diversified in parallel with them. In the specific case of *Mimosa*, it is estimated to have emerged at approximately 30 mya, and then to have radiated within a number of centers in the Americas, but most notably in the Cerrado and Caatinga biomes of Brazil, resulting in >200 species residing within them (Simon et al., 2011), many of which have been shown to be nodulated by a wide range of *Burkholderia* genotypes, some new and some already described (Bontemps et al., 2010; dos Reis et al., 2010). It is thus reasonable to suggest that the ancestral *Mimosa* spp. encountered their ancestral *Burkholderia* symbionts in these biomes, and the two partners then diversified and coevolved with each other, thus resulting in the high diversity of both symbiotic partners now evident in both biomes (Bontemps et al., 2010). Similarly, in the Cape Floristic Region (the Fynbos) of South Africa, several genera of endemic legumes in the Papilionoideae have diversified and evolved to nodulate with a group of non-*Mimosa*-nodulating *Burkholderia* spp. that possess *nod* genes that are phylogenetically closely related to *B. tuberum* STM678 (Elliott et al., 2007b; Garau et al., 2009; Gyaneshwar et al., 2011; Howieson et al., 2013). Indeed, the number of symbiotic species of *Burkholderia* has increased concomitantly as new legume symbiont diversity studies are regularly and continually being published, and this number will undoubtedly continue to increase as more centers of diversity (particularly those in the tropics and subtropics) are investigated.

Up to now, 11 species of nodulating burkholderias have been described, but several clades remain to be named (Fig. 17.1). Here is a list of the presently recognized species, together with their geographical distribution, host range, and nodulation gene origin (as shown in Fig. 17.2). This information is summarized in Table 17.1 (see also Chapter 89).

Burkholderia caribensis (Chen et al., 2003b). Two strains of this previously described non-nodulating and non-diazotrophic species have been isolated as fully functional N-fixing *M. pudica* symbionts in Taiwan. They harbor *nodA* genes closely related to *C. taiwanensis* LMG19424 (Chen et al., 2003b). However, until now this has been the only report of nodulating *B. caribensis*, and so it would appear that the occurrence of strains of this species as legume-nodulating bacteria (LNB) is rather limited.

Burkholderia diazotrophica (Sheu et al., 2012b). This species was originally found in Brazil, Taiwan, and French Guiana in nodules of "widespread" *Mimosa* species, such as *M. pudica* and *M. candollei* (syn. *M. quadrivalvis* var. *leptocarpa*) (Bontemps et al., 2010; Mishra et al., 2012), but recently it has also been isolated from nodules of *Anadenanthera* (species *peregrina* and *colubrina*), *Parapiptadenia blanchetti*, *Piptadenia viridiflora*, and *P. gonoacantha* (Bournaud et al., 2013). They carry the widespread nodC3 variant (Fig. 17.2).

Burkholderia mimosarum (Chen et al., 2006): This species seems to have an affinity for the species *Mimosa pigra* in Taiwan, Venezuela, and Brazil (Chen et al., 2005a, b, Bontemps et al., 2010), although it has also been isolated from *M. pudica* in French Guiana (Mishra et al., 2012), China (Liu et al., 2012), and India (Gehlot et al., 2013). Its type strain, PAS44, has been shown to be highly competitive for nodulation against *C. taiwanensis* LMG19424 or against various strains of alpha-rhizobia (Elliott et al., 2009).

Burkholderia nodosa (Chen et al., 2007): This species has only been found in Brazil. It is a frequent symbiont of *Mimosa* spp. (Bontemps et al., 2010) as well as *Piptadenia*, *Parapiptadenia*, and *Anadenanthera* species (Bournaud et al., 2013).

Burkholderia phenoliruptrix (Bournaud et al., 2013; de Oliveira-Cunha et al., 2012). This species was originally described on the basis of a single nonsymbiotic isolate, which was notable for its phenol-degrading properties (Coenye et al., 2004). The nodulating capability of strains from this species was first shown by Chen et al. (2005a) for strain BR3462 (syn. BR3459a), which was isolated from *M. flocculosa* in Brazil, and later sequenced and described formally as belonging to *B. phenoliruptrix* (de Oliveira Cunha et al., 2012). Several further isolates of this species were identified from the nodules of *Mimosa* and "Piptadenia Group" species, and these also proved to be true efficient nodulators of their legume hosts (Bournaud et al., 2013; clade 7 in Bontemps et al., 2010). This species harbors strains with two *nodC* variants nodC3 and nodC4 (Fig. 17.2), one being vertically transmitted (nodC4) while the other seems to have been acquired by lateral transfer (nodC3) (Bournaud et al., 2013).

Burkholderia phymatum is one of the first described species (Moulin et al., 2001; Vandamme et al., 2002). Its type strain, STM815, has been found to be an efficient symbiont of many *Mimosa* species (Elliott et al., 2007a; dos Reis et al., 2010). Among diversity studies, this species has been reported to be widely associated with *Mimosa pudica* in French Guiana (Mishra et al., 2012), China (Liu et al., 2012), and India (Gehlot et al., 2013), and as a minor symbiont of *Parapiptadenia pterosperma* in Brazil (Bournaud et al., 2013). Despite its high nodulation competitiveness ability, this species has not yet been reported among diversity studies of rhizobial symbionts of *Mimosa* species in Brazil

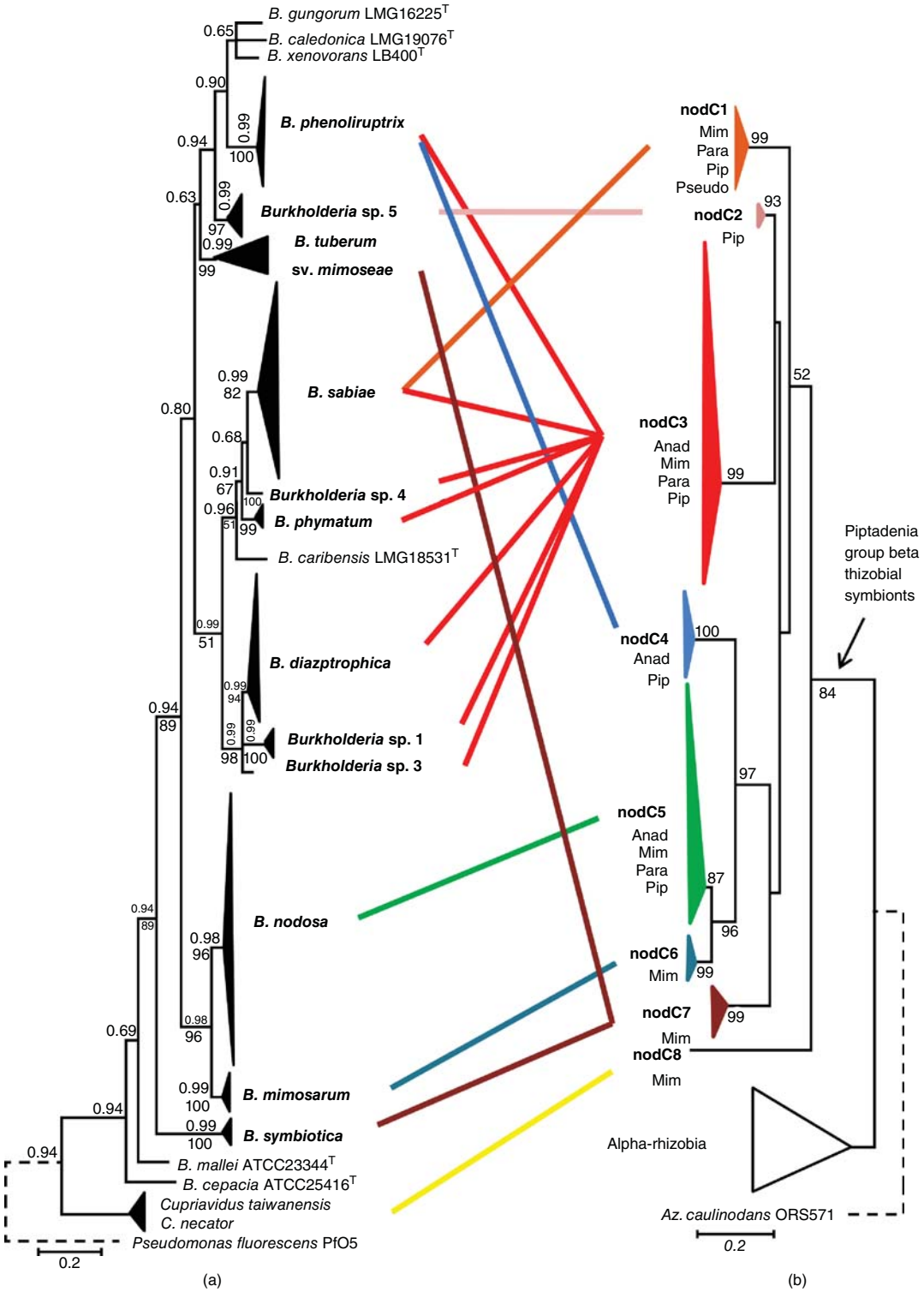

Figure 17.2 Comparison of taxonomic (a) and symbiotic (b) gene phylogenies in beta-rhizobial symbionts of the tribe Mimoseae. The figure is derived from Figure 2 of (Bournaud et al., 2013). The taxonomic marker phylogeny (a) was built using a Bayesian analysis on a partition of 16S rDNA and recA phylogenetic markers, while the symbiosis gene phylogeny was built using maximum likelihood on an alignment of the *nodC* gene. Details on analyses, strains used, and accession numbers can be found in (Bournaud et al., 2013). Legume genera associated with *nodC* clades are abbreviated as follows: Mim, *Mimosa*; Para, *Parapiptadenia*; Pip, *Piptadenia*; Pseudo, *Pseudopiptadenia*; and Anad, *Anadenanthera*.

Table 17.1 Listing of several symbiotic strains in the *Burkholderia* genus according to their species, original legume host, geographic origin, *nodC* clade (according to Fig. 17.2b), and reference

Species/Strain	Original Host	Geographic Origin	*nodC*	Reference
B. caribensis				
TJ182	*Mimosa pudica*	Taiwan	C8	Chen et al., 2003b
SWF66044	*Mimosa* sp.	China	?	Liu et al., 2012
B. diazotrophica				
JPY461T	*Mimosa quadrivilis*	Brazil	C3	Bontemps et al., 2010
SEMIA6398	*Piptadenia stipulacea*	Brazil	C3	Menna et al., 2006
BR3432	*Mimosa acutistipula*	Brazil	C3	Chen et al., 2005a
tpig4.4	*Mimosa pigra*	Costa Rica	C3	Barrett and Parker, 2006
mpp4	*Mimosa pudica*	Philippines	C3	Andrus et al., 2012
B. mimosarum				
PAS44T	*Mimosa pigra*	Taiwan	C6	Chen et al., 2005b
JPY321	*Mimosa pigra*	Brazil	C6	Bontemps et al., 2010
SWF66074	*Mimosa* sp.	China	C6	Liu et al., 2012
B. nodosa				
BR3437T	*Mimosa scabrella*	Brazil	C5	Chen et al., 2007
BR3470	*Mimosa bimucronata*	Brazil	C5	Chen et al., 2007
JPY381	*Mimosa gemmulata*	Brazil	C5	Bontemps et al., 2010
JPY301	*Mimosa setosa*	Brazil	C5	Bontemps et al., 2010
B. phenoliruptrix				
STM7454	*Anadenanthera colubrina*	Brazil	C4	Bournaud et al., 2013
STM7324	*Piptadenia paniculata*	Brazil	C4	Bournaud et al., 2013
SMF774_1	*Piptadenia monoliformis*	Brazil	C3	Bournaud et al., 2013
IIIA4A	*Anadenanthera peregrina*	Brazil	C3	Bournaud et al., 2013
JPY580	*Mimosa cordistipula*	Brazil	?	Bontemps et al., 2010
mpa10.12	*Mimosa pigra*	Australia	?	Parker et al., 2007
BR3459a	*Mimosa flocculosa*	Brazil	C3	de Oliveira Cunha et al., 2012
B. phymatum				
STM815T	*Mimosa* spp.	French Guiana	C3	Moulin et al., 2001
STM3619	*Mimosa pudica*	French Guiana	C3	Mishra et al., 2012
SWF67010	*Mimosa* sp.	China	?	Liu et al., 2012
NGR195A	*Mimosa pudica*	Papua New Guinea	C3	Elliott et al., 2007a
B. rhynchosiae				
WSM3937T	*Rhynchosia ferulifolia*	South Africa	?	De Meyer et al., 2013b
B. sabiae				
BR3407T	*Mimosa caesalpiniaefolia*	Brazil	?	Chen et al., 2005a
UYPR3.611	*Parapiptadenia rigida*	Uruguay	C1	Taulé et al., 2012
STM7373	*Parapiptadenia pterosperma*	Brazil	C1	Bournaud et al., 2013
STM7351	*Piptadenia trisperma*	Brazil	C1	Bournaud et al., 2013
Prigida2	*Parapiptadenia rigida*	Brazil	C1	Bournaud et al., 2013
STM7384	*Anadenanthera peregrina*	Brazil	C3	Bournaud et al., 2013
STM7315	*Piptadenia gonoacantha*	Brazil	C3	Bournaud et al., 2013
B. sprentiae				
WSM5005T	*Lebeckia ambigua*	South Africa	?	De Meyer et al., 2013a
B. symbiotica				
JPY345T	*Mimosa cordistipula*	Brazil	C7	Bontemps et al., 2010
JPY366	*Mimosa misera*	Brazil	C7	Bontemps et al., 2010
B. tuberum				
JPY604	*Mimosa xanthocentra*	Brazil	C7	Bontemps et al., 2010
STM3649	*Mimosa pudica*	French Guiana	C7	Mishra et al., 2012
JPY158	*Mimosa pigra*	Brazil	C7	Bontemps et al., 2010
STM678T	*Aspalathus carnosa*	South Africa	alpha	Moulin et al., 2001

Table 17.1 (*Continued*)

Species/Strain	Original Host	Geographic Origin	*nodC*	Reference
Burkholderia sp. 1				
JPY380	*Mimosa gemmulata*	Brazil	C3	Bontemps et al., 2010
JPY565	*Piptadenia viridiflora*	Brazil	C3	Bournaud et al., 2013
Burkholderia sp. 2				
mpa3.10	*Mimosa pigra*	Australia	?	Parker et al., 2007
Burkholderia sp. 3				
JPY584	*Piptadenia stipulacea*	Brazil	C3	Bournaud et al., 2013
STM7183	*Piptadenia gonoacantha*	Brazil	C3	Bournaud et al., 2013
Burkholderia sp. 4				
UYPR1.313	*Parapiptadenia rigida*	Uruguay	C3	Taulé et al., 2012
JPY268	*Mimosa pudica*	Brazil	C3	Bontemps et al., 2010
Burkholderia sp. 5				
STM7296	*Piptadenia gonoacantha*	Brazil	C2	Bournaud et al., 2013
Cupriavidus taiwanensis				
LMG19424	*Mimosa pudica*	Taiwan	C8	Chen et al., 2003b
STM6018	*Mimosa pudica*	French Guiana	C8	Mishra et al., 2012
NGR193A	*Mimosa pudica*	Papua New Guinea	C8	Elliott et al., 2007a
STM6070	*Mimosa pudica*	New Caledonia	C8	Klonowska et al., 2012
MP6	*Mimosa pudica*	India	C8	Gehlot et al, 2013
SWF66294	*Mimosa* sp.	China	C8	Liu et al., 2012
mpa3.4	*Mimosa pudica*	Costa Rica	C8	Barrett and Parker, 2006
Cupriavidus necator				
UYPR2.54	*Parapiptadenia rigida*	Uruguay	?	Taulé et al., 2012
UFLA02-71	*Phaseolus vulgaris*	Brazil	C8	Silva et al., 2012

(Bontemps et al., 2010). All strains isolated from nodules of the Mimoseae tribe harbor the nodC3 variant (Fig. 17.2).

Burkholderia rhynchosiae (De Meyer et al., 2013a). This species was isolated from *Rhynchosia ferulifolia* (tribe Phaseoleae, papilionoid) root nodules in South Africa (Garau et al., 2009). Its *nodA* genes are close to *B. tuberum* sbv *papilionoiideae*.

Burkholderia sabiae (Chen et al., 2008). This species was originally isolated from nodules of the tree "Sabiá" (*M. caesalpiniifolia*) in Brazil, but was also recently found to be a frequent symbiont of the Piptadenia Group (species of *Piptadenia*, *Parapiptadenia*, *Pseudopiptadenia*, *Anadenanthera*). The species hosts strains with two *nodC* variants (nodC1 and nodC3, Fig. 17.2).

Burkholderia sprentiae (De Meyer et al., 2013b). This species was isolated from *Lebeckia ambigua* root nodules from South Africa (Howieson et al., 2013). It is close to the species *B. tuberum*.

Burkholderia symbiotica (Sheu et al, 2012a). This species has only been reported in Brazil in nodules of the Caatinga endemics *Mimosa cordistipula* and *M. misera* (Bontemps et al., 2010; Sheu et al., 2012a). Members of this species carry the nodC7 variant.

Burkholderia tuberum (Vandamme et al., 2002) is a species belonging to a large clade of related symbionts that display two large symbiovars with distinct nodulation gene origins (Mishra et al., 2012): "sv. mimoseae" for symbionts of legumes in the tribe Mimoseae (Bontemps et al., 2010; Mishra et al., 2012), all carrying the nodC7 variant (Fig. 17.2); and "sv. papilionoideae" for South African papilionoid endemics (Howieson et al., 2013), carrying nodulation genes closer to alpha-rhizobia (Chen et al., 2003b). These symbiovars are not yet taxonomically approved, but, nevertheless, can be used to distinguish the two host range and nodulation gene origins. The *B. tuberum* "sv. *mimoseae*" strains have been reported only in the nodules of *Mimosa* species in Central and South America, that is, French Guiana (Mishra et al., 2012), Brazil (Bontemps et al., 2010), Mexico (Ormeno-Orrillo et al., 2012), Panama (Barrett and Parker, 2005), and Costa Rica (Barrett and Parker, 2006). They carry the nodC7 variant, which is only found in *Mimosa*-nodulating strains. For the *B. tuberum* sv. papilionoideae, evidence of effective nodulation on *Macroptilium atropurpureum* (Angus et al., 2013) and *Cyclopia* species (Elliott et al., 2007b) has been published. The large *Burkholderia* clade that includes *B. tuberum* clearly needs further taxonomic revision as it very likely contains more than one species. On this topic, a new species has already been defined as *B. sprentiae* (De Meyer et al., 2013b).

17.2.1 Unnamed New Species

Several strains of *Burkholderia* isolated from nodules of various members of the tribe Mimoseae probably belong to new species (see *Burkholderia* sp. 1–5 in Figs. 17.1 and 17.2; see also Chapter 89). This is the case with four clades of strains (*Burkholderia* sp. 1, 3, 4, 5) isolated in Brazil from *Piptadenia* and *Parapiptadenia* species (Bournaud et al., 2013), of which some are currently the focus of a taxonomic study aimed at describing a new species (Bournaud et al., unpublished), together with strains isolated from *M. pudica* in Australia (*Burkholderia* sp. 2, (Parker et al., 2007)). Other strains isolated from papilionoid legumes of the Fynbos in South Africa (in the genera *Rhynchosia*, *Lebeckia*) also probably belong to new species (Garau et al., 2009; Howieson et al., 2013).

17.2.2 Burkholderia: Toward the Borders between Mutualism and Pathogenicity?

Interestingly, no members of the phytopathogenic/pathogenic *Burkholderia* group (including the "*B. cepacia* complex") have, so far, been found to nodulate legumes, although they are frequently isolated from them as "passengers" or "endophytes" (Gyaneshwar et al., 2011; Suarez-Moreno et al., 2012). The only isolate of *B. cepacia* described as a nodule symbiont was isolated from a *Dalbergia* species in Madagascar (Rasolomampianina et al., 2005), but its nodulating ability could not be clearly established and the *nod* and *nif* genes could not be amplified from this strain (L. Moulin, unpublished data). Attempts to separate the two *Burkholderia* clades (pathogenic/nonpathogenic) are being undertaken, based either on multilocus sequence analyses (MLSA) or on different phenotypic tests, as well as their propensity for pathogenicity (Estrada-de los Santos et al., 2013; see Chapter 89). In spite of their proven potential for practical applications in agriculture (Suarez-Moreno et al., 2012), the use of *Burkholderia* strains for agronomy or ecology is currently not widely permitted owing to the presence of human and plant pathogens within the genus. It is, therefore, hoped that the establishment of the exact genetic boundaries between the pathogenic and nonpathogenic clades, and possibly the separation of them into two genera (Gyaneshwar et al., 2011), will assist in allowing the use of the mutualistic *Burkholderia* for plant growth improvements in agronomy and ecological restoration (see also Chapter 89).

17.3 ORIGIN AND DIVERSITY OF *Cupriavidus* LEGUME SYMBIONTS

Cupriavidus is closely related to *Burkholderia*, but unlike the latter genus it contains very few N-fixing species. The genus was redefined by Coenye et al. (2004), and several species from the genus *Ralstonia* were included in it, including (at that time) the sole diazotrophic *Ralstonia* species, *R. taiwanensis*. *Cupriavidus taiwanensis* has now been isolated from nodules on invasive *M. pudica* and *M. diplotricha* in many countries in SE Asia (China, India, Taiwan, Philippines, Table 17.1) and Australasia (Papua New Guinea, New Caledonia), and it may often predominate as symbionts of these *Mimosa* spp. in these countries (Chen et al., 2001, 2003a, b; 2005b; Elliott et al., 2009; Liu et al., 2012; Andrus et al., 2012; Klonowska et al., 2012; Gehlot et al., 2013). The predominance of *C. taiwanensis* in some environments appears somewhat surprising, as *M. pudica* and *M. diplotricha* also have the potential to nodulate with *Burkholderia*, and the latter will outcompete *C. taiwanensis* in laboratory-based experiments (Elliott et al., 2007a, 2009). On the other hand, under particular soil conditions, such as when N-levels are increased from close to zero, the competiveness of *C. taiwanensis* is increased relative to *Burkholderia* (Elliott et al., 2009), and this most likely explains why it is frequently found in nodules on *Mimosa* growing in high(er) fertility neutral-alkaline soils (Mishra et al., 2012; Liu et al., 2012; Gehlot et al., 2013). In addition to this, however, and as suggested by its name, an additional factor that can contribute to the competitive ability of *C. taiwanensis* in some environments (e.g., in New Caledonia) is its presence in the soil of high concentration of metals, particularly Cu and Zn, and also Fe (Chen et al., 2008; Klonowska et al., 2012).

So from where does *C. taiwanensis* originate? Although it is found throughout much of SE Asia and Australasia, both *M. pudica* and *M. diplotricha* are invasive plants that were taken to these environments over the last 500 years by European colonists from tropical coasts of the newly discovered New World (Chen et al., 2005b). This means that the symbionts of these plants in these invasive environments almost certainly came with them from the Americas, and this has been shown clearly for both *Burkholderia* spp. and *C. taiwanensis* (Chen et al., 2005a, b; Andam et al., 2007; Parker et al., 2007; Andrus et al., 2012), but where in the Americas did it originally evolve its nodulating ability? Based on its ability to nodulate *Mimosa*, the first places to look might be the centers of *Mimosa* radiation, such as the Cerrado and the Caatinga in Brazil, but, surprisingly, no *Cupriavidus* symbionts were found during the studies of Bontemps et al. (2010) and dos Reis et al. (2010), which suggests that they have a different center of origin to the *Mimosa*-nodulating burkholderias, and that unlike *Burkholderia* they may not have coevolved with *Mimosa*. Indeed, this is supported by the fact that *C. taiwanensis* has a much reduced host range in the genus *Mimosa* when compared to *B. phymatum* (Elliott et al., 2007a), and also by the fact that most Brazilian species (including Cerrado/Caatinga endemics) cannot nodulate effectively (or at all in some cases) with

C. taiwanensis (Elliott et al., 2007a; dos Reis et al., 2010). This reduced host range within the genus *Mimosa* might be linked to *C. taiwanensis* being a young symbiont (Amadou et al., 2008) relative to the *Mimosa*-nodulating burkholderias, which probably acquired their nodulating ability >50 mya (Bontemps et al., 2010). It is possible that *C. taiwanensis* recently acquired its symbiotic ability from promiscuous *Mimosa*-nodulating burkholderias that were spread with invasive/widespread *Mimosa* spp. (such as *M. pudica*) throughout tropical and subtropical South America (Andam et al., 2007; Gyaneshwar et al., 2011; Silva et al., 2012), but most particularly in the coastal lowlands where they predominate (Mishra et al., 2012).

On the other hand, there is a conflicting pattern for the origin of *nod* genes in *Cupriavidus* between *nodA* and *nodC* phylogenies. In the *nodA* phylogeny, *Cupriavidus* seems to have recently acquired a *nodA* gene from the ancestor of the *B. phymatum/B. sabiae* clade (Mishra et al., 2012), while in the *nodC* phylogeny *Cupriavidus* symbionts are outgrouped from all *Burkholderia*–Mimoseae symbionts, forming a separate clade (nodC8, Fig. 17.2b), and thus suggesting a much more ancient transfer of *nod* genes than the *nodA* phylogeny. The origin of nodulation in *Cupriavidus* could thus be more ancient than previously expected. This is supported by the recent description of other species of *Cupriavidus* that can nodulate legumes, such as *C. necator* and *C. necator*-like strains that have been shown to nodulate *Mimosa* spp. (and other legumes) in Brazil and Uruguay (da Silva et al., 2012; Taulé et al., 2012, Table 17.1). Interestingly, although the *C. necator* strains from da Silva et al. (2012) were isolated during soil "trapping" experiments with promiscuous legume hosts (*Leucaena leucocephala*, *Phaseolus vulgaris*) and, therefore, are not directly linked at this time to any particular host, their *nod* gene (*nodA*, *nodC*) and *nifH* sequences are similar to those of *C. taiwanensis* LMG19424^T and other *Mimosa*-nodulating beta-rhizobial strains (Fig. 17.2b), which suggests that their actual host(s) might also be *Mimosa* spp. Indeed, the soil used for the trapping experiments came from the south of the state of Minas Gerais (MG) (Florentino et al., 2012), and hence was within the endemic range of *Mimosa* in Brazil (Simon et al., 2011; dos Reis et al., 2010), but the soil used to trap the *C. necator* strains of da Silva et al. (2012) was not similar to Cerrado soil. In fact, its pH was neutral-alkaline rather than the highly acidic pH, which is more typical of Cerrado soils. It is known that *Mimosa*-nodulating *Burkholderia* generally prefer acidic soils, whereas symbiotic *Cupriavidus* prefer neutral-alkaline soils (Elliott et al., 2009; Mishra et al., 2012), so it is possible that *Mimosa* spp. that are endemic to neutral-alkaline non-Cerrado soils in MG are preferentially nodulated by *Cupriavidus* spp. Additional factors that may potentially favor *Cupriavidus* in the soils of the metal-mining state of MG are the presence of high levels of heavy metals, particularly of Zn and Cu (Chen et al., 2008; Ferreira et al., 2012; Klonowska et al., 2012).

Therefore, all the available information about the distribution of legume-nodulating *Cupriavidus* suggests that when considering where to search for the origins and/or the original hosts of *Cupriavidus* in tropical and subtropical America, two types of environments should be focused on (i) coastal lowlands and/or "disturbed" environments with neutral-alkaline soils with relatively high fertility, such as wasteground, where ruderal species, such as *M. pudica* and *M. diplotricha*, are dominant weeds and (ii) nonacidic soils anywhere in the native range American *Mimosa* (i.e., southern United States to northern Argentina; Simon et al., 2011) that have relatively high natural levels of Cu and Zn (and possibly also Fe). In the latter case, a strong possibility that is now emerging as a candidate for a center of *Cupriavidus* diversity is Uruguay (Battistoni, Fabiano, Cold, Taulé, unpublished), where there are several endemic *Mimosa* spp. that appear to be nodulated by *Cupriavidus* strains related to the *C. necator*-like strains that were isolated from *Parapiptadenia rigida* in the same geographical region (Taulé et al., 2012). These include *M. uruguensis*, which has previously been shown to nodulate effectively with *C. taiwanensis*, but not *B. phymatum* STM815 (Elliott et al., 2007a). Further work is being conducted to determine if Uruguayan *Mimosa* spp. are preferentially nodulated by *Cupriavidus*, and also if there are soil conditions that may have driven their coevolution.

On another aspect, *C. taiwanensis* pSym is used in experimental evolution studies aiming at turning a plant pathogen (*Ralstonia solanacearum*) into a legume symbiont (Marchetti et al., 2010; Guan et al., 2013).

17.4 NEW RHIZOBIAL TAXA IN THE BETA AND GAMMA SUBCLASSES OF PROTEOBACTERIA?

At present, the only confirmed LNB outside the Alphaproteobacteria belong to the closely related Betaproteobacterial genera *Burkholderia* and *Cupriavidus* (Gyaneshwar et al., 2011), but there have been many claims over the last 20 years for other "exotic" LNB. One possible candidate is *Herbaspirillum* (see Chapter 93). Both *Burkholderia* and *Cupriavidus* are related to it (Tayeb et al., 2008; Hassen et al., 2012), and it is known as a genus that has diazotrophic species that associate closely (often endophytically) with several nonlegumes and also promote their growth (Monteiro et al., 2012). Interestingly, plant-associated herbaspirilla are also found associated with legumes, particularly common bean (Schmidt et al., 2011; Monteiro et al., 2012), and have even been isolated from within their nodules (Valverde et al., 2003). However, convincing evidence that *Herbaspirillum* can actually nodulate legumes is still lacking, and there

is no indication from any of the genomes that have been sequenced so far that herbaspirilla have this capacity. For example, *H. lusitanum*, the species isolated from common bean nodules by Valverde et al. (2003), does not even have any N-fixation genes in its genome (Weiss et al., 2012), and although *H. seropedicae* can clearly fix N it is not known to harbor *nod* genes (Pedrosa et al., 2011). Nevertheless, the possibility that herbaspirilla might nodulate legumes has recently received a boost by the recent report by Hassen et al. (2012) of two *Herbaspirillum* strains that were isolated from the South African legume *Aspalathus linearis* (Papilionoideae), and which can also apparently nodulate this plant and fix N.

There have also been reports of other Betaproteobacterial genera, such as *Achromobacter* and *Janthinobacterium*, as being potentially effective nodulators of legumes (Benata et al., 2008; Lima et al., 2009; Azarias Guimaraes et al., 2012). Similarly, in the Gammaproteobacteria, there are claims of nodulating strains of *Pseudomonas* (Shiraishi et al., 2010; Huang et al., 2012) and *Stenotrophomonas* (Lima et al., 2009). However, it should be noted that in all these cases, including *Herbaspirillum* (Hassen et al., 2012), Kock's postulates appear to have not yet been fulfilled, that is, the inoculated strains have not been reisolated from the nodules and/or there is no microscopical evidence confirming their occupation of the infected cells of the nodules in a bacteroid form. Highly rigorous tests of the ability of several nodulating *Burkholderia* and *Cupriavidus* strains were performed before they were accepted as LNB (see Introduction), and in consideration that nodules are an excellent endophytic niche for many opportunistic nonsymbiotic bacteria (Gyaneshwar et al., 2011), it is reasonable to expect similarly rigorous tests to be applied to *Herbaspirillum*, *Achromobacter*, *Pseudomonas*, etc., before any of these "exotic" Beta- and Gammaproteobacteria can be fully accepted as being LNB.

17.5 HAS *Mimosa* COEVOLVED WITH ITS SYMBIONTS?

An earlier attempt at examining the symbiont preferences of *Mimosa* spp. in the context of the taxonomy of this large and complex legume genus broadly concluded that the Brazilian and South American species had an ability to nodulate effectively with the promiscuous beta-rhizobial strain *B. phymatum* STM815, whereas the Mexican species did not (Elliott et al., 2007a). Since then, a number of studies on *Mimosa* spp. and/or their symbionts have been undertaken (see also Chapter 89). First, Simon et al. (2011) have published a comprehensive molecular phylogeny using the *trnD-trnT* plastid sequences from 259 *Mimosa* spp., which has confirmed that the Brazilian and Mexican spp. are phylogenetically separate, and that they are grouped in several clades consisting of many closely related and highly endemic species that are confined to particular biomes, such as the Cerrado and Caatinga of Brazil and the Central Mexican highlands. Second, Bontemps et al. (2010) have shown that the symbionts of c. 47 of the Cerrado/Caatinga native and endemic *Mimosa* spp. are almost exclusively *Burkholderia*, and third, a study of several central Mexican endemic *Mimosa* spp. has shown that they are nodulated by Alphaproteobacteria in the genera *Rhizobium* and *Ensifer* (syn. *Sinorhizobium*) (Bontemps and Rogel, unpublished). Concomitantly with these diversity studies, it has also been demonstrated that the Cerrado/Caatinga endemics are not able to be nodulated effectively (or at all) by *B. phymatum* STM815 (dos Reis et al., 2010).

These data, taken together with those of Elliott et al. (2007a, 2009), suggest that the central Brazilian and central Mexican endemic *Mimosa* spp. have adopted very different symbionts, and that they are not capable of nodulating with each other's symbionts. This strongly suggests some degree of coevolution of these two geographically and taxonomically very separate groups of *Mimosa* spp. with their respective rhizobial symbionts. It is not clear about how and when the Brazilian and Mexican endemic *Mimosa* spp. became geographically separated from each other (Simon et al., 2011), but a reasonable scenario is that the ancestors that gave rise to these endemic *Mimosa* lineages reached central Brazil and central Mexico, where in both locations they became adapted to highland regions with poor soils (low N, low organic matter). In the case of Brazil, the soils will have been acidic and will have had a population of potential symbiotic bacteria that were adapted to those conditions (i.e., *Burkholderia* spp.), whereas in Mexico the soils will have been neutral/alkaline and housed Alphaproteobacterial symbionts (*Ensifer*, *Rhizobium*). Over evolutionary time, each group of mimosas will have radiated and speciated, resulting in a high level of endemicity in highland biomes in both countries; this endemicity will be related to many factors, including altitude (Simon and Proença, 2000). Given that each endemic species will, by definition, have been confined to a particular locality, it is likely that they will also have been obliged to adopt their rhizobial symbionts from that specific locality, be they *Burkholderia* (Brazil) or *Ensifer*/*Rhizobium* (Mexico). As they became increasingly cut off from their neighbors, each endemic *Mimosa* sp. and its symbiont will have evolved together, but separately, not only from their immediate neighbors, but also from their "widespread" relatives in the lowlands. Thus, we now see today in central Brazil, a set of highly endemic *Mimosa* spp. that have evolved over the last 10 my at altitudes >1000 m (Simon et al., 2011), and which have *Burkholderia* symbionts with a genotype that is also found only at these altitudes (Bontemps et al., 2010). The possibility that this is also the case with the (*Ensifer*/*Rhizobium*) symbionts of the Mexican highland endemics is currently being investigated

(Bontemps and Rogel, unpublished), but it is likely that the Mexican endemics are also nodulated by particular nod genotypes.

Although Brazil and Mexico contain the largest centers of radiation of the genus, *Mimosa* has other centers of endemicity in both the New World (e.g., Uruguay and subtropical South America, the Andes) and the Old World (India, Madagascar) (Simon et al., 2011; see Chapter 89). Little is so far known about these other centers, except that (as we have previously mentioned) Uruguayan endemics may be preferentially nodulated by *Cupriavidus* spp., and that two Indian species, *M. hamata* and *M. himalayana*, although they have only recently (6–10 mya) been separated from their central Brazilian relatives (Simon et al., 2011), are not nodulated by *Burkholderia*, but by *Ensifer* strains that are closely related to the symbionts of the "local" mimosoid legumes (Gehlot et al., 2013). This latter study further illustrates, along with the Brazilian highland endemics (Bontemps et al., 2010), that geographical isolation, even for a relatively short period (<10 my), of a legume population in a region whose soil is populated by a particular symbiont type will eventually oblige these legumes (in these cases *Mimosa* spp.) to form a symbiosis with them. Over time, continued isolation of both symbiotic partners will, inevitably, lead to their coevolution without any significant input from horizontal gene transfer (at least in the case of the bacterial partner).

17.6 IS THERE EVIDENCE OF COEVOLUTION BETWEEN LEGUMES IN THE TRIBE MIMOSEAE AND THEIR *Burkholderia* SYMBIONTS?

In the wider context of the tribe Mimoseae and the Piptadenia Group, to which *Mimosa* belongs (Jobson and Luckow, 2007), it is difficult, however, to discern any solid relationships between the various legume genera and the symbionts that have so far been isolated from them, except that, in general they have a preference for nodulating with *Burkholderia* strains that are similar to those that nodulate Brazilian and generally "Widespread" *Mimosa* spp. (Bournaud et al., 2013). So, although burkholderias have been shown to be diversified symbionts of legume species of the tribe Mimoseae, and that the onset of diversification of the plant and *Burkholderia* species might have occurred concomitantly (ca. 30 mya), no strict pattern of coevolution appears when plant and *Burkholderia* phylogenies are compared (Bournaud et al., 2013; see also Chapter 89). This pattern, if it has ever existed, is either not discernible owing to site effects and/or it has been disrupted by the broad range of partners chosen by both the legumes and the *Burkholderia* species, for example, *B. nodosa* and *B. sabiae* are found as symbionts of almost every genus and species of the Piptadenia Group, including *Mimosa*.

Although nodulation genes in *Burkholderia* strains isolated from *Mimosa* species have been shown to be vertically inherited (Bontemps et al., 2010), as more legume genera in the tribe Mimoseae are found to be nodulated by beta-rhizobia, this vertical pattern of nodulation gene transfer in mimoseae–burkholderias shows an increasingly more complex pattern (Fig. 17.2b). Some *nodC* variants (such as nodC3) are much more frequent within species than others, while other species (such as *B. phenoliruptrix* and *B. sabiae*) carry two *nodC* variants, indicating that horizontal transfer also played a role in the diversification of symbiosis in *Burkholderia* (Bournaud et al., 2013). It is now well established that horizontal gene transfer tends to erase evidence of coevolutionary relationships between plant radiation and speciation in rhizobia (Martinez-Romero, 2009), and this phenomenon can also be observed for beta-rhizobia. No strict correlation can be found between *nodC* clades and the host range or symbiotic ability of the burkholderias, as most *nodC* variants were found in strains nodulating the same genera and species. On the other hand, some *nodC* clades were found only in *Mimosa*-nodulating symbionts (nodC6, C7, C8, Fig. 17.2b), indicating possible host specificity, but this was only identified because *Burkholderia* strains were isolated from several endemic *Mimosa* species that had been separated from their widespread relatives for >10 my (see previous section). Clearly, more host range studies of strains from each species and carrying the different *nodC* variants are required to get a better idea of host specificity in this group of symbionts. In addition, although none of the other Piptadenia Group genera are anywhere near as rich in species as *Mimosa*, and some are also paraphyletic (e.g., *Piptadenia* s.l., and perhaps *Stryphnodendron*) (Jobson and Luckow, 2007), which hinders our ability to investigate them as deeply as it has been possible to do with a large monophyletic genus like *Mimosa*, it may be that further investigations within the Group, particularly into the symbionts of highly endemic species that are specific to very particular environments, will result in the identification of more specifically-related (and even novel) symbiotic genotypes.

17.7 CONCLUSION

Twelve years after the first description of beta-rhizobia, the biodiversity of this group of symbionts is emerging as highly diversified in several groups (especially in *Burkholderia*) with high affinities with legume species in the tribe Mimoseae and in several South African endemic species in the Papilionoideae. The-root nodulating ability of Betaproteobacteria may also be extended to new genera, such as *Herbaspirillum* and *Achromobacter*, although more data on their nodulation abilities are required to establish their status as true symbionts. As more biodiversity surveys of

symbionts of endemic legume species are achieved, the wider picture (including the possibility of host coevolution) of the beta-side of the extended rhizobial family will be revealed (see also Chapter 89).

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Chapter 18

Bradyrhizobium, the Ancestor of All Rhizobia: Phylogeny of Housekeeping and Nitrogen-Fixation Genes

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18.1 INTRODUCTION

The widespread use of legumes (family Leguminosae = Fabaceae) as food crops, forages, and green manures is mainly associated with their ability to establish symbiotic associations with nodulating nitrogen-fixing bacteria collectively known as rhizobia. These bacteria have been subject of many studies, with an emphasis on their key role in the replacement of chemical N fertilizers, improving agricultural sustainability – especially in tropical nitrogen-impoverished soils – with profound environmental impacts, including lower emission of greenhouse gases and pollution of water by nitrate. One outstanding example is the case of the soybean (*Glycine max* [L.] Merr.) crop, discussed in Chapter 99. However, despite the global economic, environmental, and social importance, in comparison with other groups of bacteria, for example, human, plant, and animal pathogens, few studies have been performed on the taxonomy, phylogeny, and genomics of rhizobia (see Chapter 3).

The family Leguminosae encompasses over 18,000 species, classified into around 650 genera, occupying nearly all terrestrial biomes (Polhill and Raven, 1981). Rhizobia have been isolated from root nodules predominantly in the subfamilies Mimosoideae and Papilionoideae; root nodules are rare in the Caesalpinioideae (Allen and Allen, 1981). Unfortunately, still today information about nodulation capacity is lacking for nearly half of the genera of Leguminosae (Sprent, 2009). However, in comparison with the number of legumes known to nodulate, very few rhizobial species have been described – about a hundred (DSMZ, 2013). Nitrogen-fixing rhizobia belonging to the genus *Bradyrhizobium* are abundant in many soils, particularly in the tropics (e.g., Moreira et al., 1993; Menna et al., 2006, 2009a), and have been isolated from the nodules of highly divergent legume tribes, including herbaceous and woody species of tropical and temperate origins, aquatic legumes such as *Aeschynomene*, and the nonlegume *Parasponia andersonii* (Trinick, 1973; Sprent, 2001; Menna et al., 2006). Also, it has been isolated as an endophyte of nonlegumes (e.g., Chaintreuil et al., 2000; Tan et al., 2001) and detected in a variety of environmental metagenomes that have been published, confirming the broad distribution of the genus.

Bradyrhizobium represents the ancestor of all rhizobia (e.g., Lloret and Martínez-Romero, 2005); even though, the genus is far less studied than *Rhizobium* – probably due to its intrinsic physiological properties, with an emphasis on the slow growth rate *in vitro* that makes it more difficult to conduct genetic studies. In this chapter, we review phylogenetic insights into nitrogen-fixing *Bradyrhizobium*, highlighting results obtained by our group.

18.2 STATE OF THE ART OF THE GENUS *Bradyrhizobium*

Long before the nitrogen-fixation process was elucidated, the benefits of the biological process were recognized.

Biological Nitrogen Fixation, Volume 1, First Edition. Edited by Frans J. de Bruijn.

The Romans developed the idea of crop rotation with legumes and nonlegume plants to improve soil "health" and "quality." In 1813, interest in chemistry led Sir Humphrey Davy to report that the legumes "seemed to prepare the ground for the wheat," and he speculated that the nitrogen came from the atmosphere. At that time, it was a common practice to transfer soil from a field where legumes had been grown to areas being newly planted (e.g., to new pastures of alfalfa – *Medicago sativa* L. – in England), representing the first inoculations. In 1838, Boussingault, a French agricultural chemist showed that legumes had higher N levels than cereals and concluded that the atmosphere was the source. Unfortunately, in 1848, Liebig, an internationally eminent chemist, argued that the source of N was ammonia and not the atmosphere. Finally, in 1886, German scientists, Hellriegel and Wilfarth, demonstrated that the ability of legumes to convert N_2 from the atmosphere into compounds that could be used by the plant was due to the presence of swellings or nodules on the roots and of particular bacteria within these nodules (reviewed in Voelcker, 1896; Fred et al., 1932; Cooper, 2003; Hungria and Campo, 2004; Hungria et al., 2005; Ormeño-Orrillo et al., 2012; see also Chapter 88).

Shortly after, in 1888, the Dutch microbiologist Martinus Willem Beijerinck isolated bacteria from nodules of pea (*Pisum sativum* L.), which were termed *Bacillus radicicola*, and demonstrated that they had the ability to reinfect the host legume and fix nitrogen in symbiosis (Beijerinck, 1888, 1890). In 1890, German scientists, Nobbe and Hiltner, demonstrated the advantages of adding pure bacteria with the seed at sowing, and the discovery was patented, resulting in the first commercial product (Voelcker, 1896); we now understand that this inoculant for soybean carried bradyrhizobia.

Some 40 years later, a first systematic classification of nitrogen-fixing rhizobia was proposed, based on the cross-inoculation concept with respect to the host legume, resulting in six species of *Rhizobium*: *R. meliloti*, *R. trifolii*, *R. phaseoli*, *R. lupini*, *R. leguminosarum*, and *R. japonicum* (Fred et al., 1932).

Fifty years later, the taxonomy was redefined using numerical criteria, including several phenotypic and genetic properties. A new genus was created, *Bradyrhizobium* (*bradus*, from the Greek, meaning slow, referring to the growth rate *in vitro*) to accommodate rhizobia with the typical properties of alkaline reaction and slow growth rate in culture media containing mannitol as carbon source (Jordan, 1982). However, it is interesting to remember that the division of rhizobia into fast and slow growers *in vitro* had been proposed much earlier by Löhnis and Hansen (1921).

In relation to the evolution of rhizobia, in 1965 Norris suggested that *Bradyrhizobium* was the ancestor of alpha-rhizobia and originated in the tropics (Norris, 1965). Several decades later, studies with new molecular tools confirmed Norris's hypothesis (Provorov and Vorob'ev, 2000; Lloret and Martínez-Romero, 2005). Ancient and conserved molecules have been used as molecular clocks to estimate time divergence between lineages in response to changes that have occurred in their sequences. The ancestral gene of glutamine synthetase (GS) doubled before the separation of Prokaryotes and Eukaryotes (Pesole et al., 1995), and the comparison between the two paralogous genes GSI and GSII allowed estimates of the times of divergence between lineages of Alphaproteobacteria (Turner and Young. 2000; see Chapter 3). From this comparison, estimates for the two genes were similar in fast-growing rhizobia, and *Bradyrhizobium* was highlighted as the ancestor of all rhizobia. Interestingly, *Bradyrhizobium* is the only rhizobial genus that has preserved the ability to photosynthesize (see Chapter 28) and, together with *Azorhizobium*, can fix nitrogen both in free-living and in symbiotic conditions.

18.3 DESCRIBED SPECIES OF *Bradyrhizobium*

Initially, *Bradyrhizobium japonicum* was the only described species for the genus, encompassing symbionts of soybean; for symbionts of other legumes, the nomenclature used was *Bradyrhizobium* sp. followed by the host plant name (Jordan, 1982, 1984). The reports of large genetic and physiological variability among strains that nodulate soybean led to the description of *Bradyrhizobium elkanii* a few years later (Kuykendall et al., 1992).

Thereafter, other species were described, including symbionts of legumes other than soybean: *Bradyrhizobium yuanmingense*, symbiont of *Lespedeza* spp., a legume that grows in the northern hemisphere (Yao et al., 2002); *Bradyrhizobium canariense*, a symbiont of genistoid legumes from the Canary Islands (Vinuesa et al., 2005); *Bradyrhizobium pachyrhizi* and *Bradyrhizobium jicamae*, isolated from effective nodules of *Pachyrhizus erosus* (L.) Urb., commonly called jicama or Mexican yam and known for its tuberous roots (Ramírez-Bahena et al., 2009); *Bradyrhizobium cytisi* (Chahboune et al., 2011) and *Bradyrhizobium rifense* (Chahboune et al., 2012), isolated from effective nodules of *Cytisus villosus* Pourr. (a legume with broad distribution in the Mediterranean and northern Africa) grown in soils of Morocco; *Bradyrhizobium lablabi*, isolated from effective nodules of *Lablab purpureus* (L.) Sweet and peanut (*Arachis hypogaea* L.) in Anhui and Sichuan provinces of China (Chang et al., 2011); and *Bradyrhizobium arachidis*, also isolated from peanut in China (Wang et al., 2013b). There is also the reclassification of *Blastobacter denitrificans as Bradyrhizobium denitrificans*, for the aquatic symbiont of *Aeschynomene indica* (van Berkum et al., 2006, 2011).

In relation to the soybean, new species from China have been described: *Bradyrhizobium liaoningense* has been defined as an extra-slowly growing *Rhizobium* that nodulates primitive and modern soybean genotypes (Xu et al., 1995), and *Bradyrhizobium huanghuaihaiense* (Zhang et al., 2012) *and Bradyrhizobium daqingense* (Wang et al., 2013a), both effective nodulators of soybean. In addition, a new species has been recently described – *Bradyrhizobium diazoefficiens* – for the strains previously classified as *B. japonicum* group Ia (Delamuta et al., 2013).

The genus *Bradyrhizobium* also includes non-nodulating nonsymbiotic bacteria. *Bradyrhizobium betae* refers to endophytes isolated from sugar beet (*Beta vulgaris* L.) roots affected by tumor-like deformations (Rivas et al., 2004), and *Bradyrhizobium iriomotense* has also been isolated from a tumor-like root of the legume *Entada koshunensis* Hayata and Kaneh in Japan (Islam et al., 2008). There is also the reclassification of *Agromonas oligotrophica* into *B. oligotrophicum*, a nitrogen-fixing oligotrophic bacterium isolated from paddy soils in Japan (Ramírez-Bahena et al., 2013).

We may conclude that, despite *Bradyrhizobium* being the predominant symbiont of a variety of tropical legumes (e.g., Moreira et al., 1993; Menna et al., 2006, 2009a), in addition to the suggestion that diversity should be greater in tropical than in temperate regions (e.g., Oyaizu et al., 1992), in early 2013, only 15 species of *Bradyrhizobium* have been described as symbionts of legumes. This reinforces the suggestion that *Bradyrhizobium* diversity in the tropics is poorly documented (Zakhia and de Lajudie, 2001; Menna et al., 2006, 2009a, 2009b), and that hundreds of *Bradyrhizobium* species remain to be described (e.g., Willems et al., 2001a; Menna et al., 2006, 2009a, 2009b).

18.4 PHYLOGENY BASED ON THE ANALYSIS OF RIBOSOMAL GENES

Ribosomal sequences – with an emphasis on 16S rRNA – have become the basis of bacterial molecular phylogeny and taxonomy (Woese, 1987; Weisburg et al., 1991; Olsen and Woese, 1993; Willems and Collins, 1993; Garrity and Holt, 2001; see Chapter 3). Based on 16S rRNA (e.g., Wang and Martínez-Romero, 2000) and also on genomic analyses (e.g., Carvalho et al., 2010), rhizobia are currently positioned in four taxonomic branches: *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium–Sinorhizobium* (=*Ensifer*). These branches include nonsymbiotic relatives (oligotrophic soil and aquatic bacteria such as *Rhodopseudomonas palustris*, *Rhodoplanes roseus*, *Nitrobacter winogradskyi,* and *B. denitrificans*, and pathogens such as *Afipia* spp.), which may indicate common ancestry for rhizobial species and other parasitic and soil-borne bacteria.

Since the 1990s and early 2000s, partial or complete 16S rRNA sequences have been extensively applied to phylogeny studies of *Bradyrhizobium* (e.g., Young et al., 1991; Oyaizu et al., 1992; Yanagi and Yamasato, 1993; van Rossum et al., 1995; Urtz and Elkan, 1996; Moreira et al., 1998; Vinuesa et al., 1998; Wang et al., 1999; Chen et al., 2000; Jarabo-Lorenzo et al., 2000; Willems et al., 2001b; see Chapter 3). However, all of these, in addition to other later studies, have shown that despite a high level of diversity in morphological, physiological, and genetic properties (e.g., van Rossum et al., 1995; Moreira et al., 1993; So et al., 1994; Boddey and Hungria, 1997; Doignon-Bourcier et al., 1999; Chen et al., 2002), the diversity in the 16S rRNA sequences of *Bradyrhizobium* is low and distinguishes species poorly (e.g., Menna et al., 2006, 2009a; Delamuta et al., 2013). A phylogenetic tree of *Bradyrhizobium* species, based on 16S rRNA, is shown in Figure 18.1.

Other ribosomal genes can add information about phylogeny that helps to clarify the taxonomy of *Bradyrhizobium* species. The 23S rRNA is a long fragment of about 2.3 kb; it contains more information than 16S rRNA and has proven to be useful in the definition of several genera and species of bacteria (Ludwig and Schleifer, 1994) including rhizobia (Tesfaye et al., 1997; Terefework et al., 1998; Tesfaye and Holl, 1998; Qian et al., 2003; Germano et al., 2006). Furthermore, because the rate of sequence change seems to be faster in 23S rRNA than in 16S rRNA (Olsen and Woese, 1993), the former allows a finer definition of phylogenetic relationships

Analysis of the 16S–23S rRNA intergenic transcribed spacer (ITS) (also called intergenic spacer IGS) has proven to be useful in studies of phylogeny and taxonomy of *Bradyrhizobium*. Most bradyrhizobia carry only one copy of ITS; the sequence is long and with higher variability, making the region particularly useful for elucidation of phylogeny and taxonomy (Laguerre et al., 1996; Vinuesa et al., 1998; Doignon-Bourcier et al., 2000; van Berkum and Fuhrmann, 2000; Parker and Lunk, 2000; Willems et al., 2001a; Germano et al., 2006; Menna et al., 2009a). Recently, the genome sequencing of *B. japonicum* USDA 6^T revealed two copies of the rRNA gene cluster [*rrn*] in the chromosome (Kaneko et al., 2011), confirmed in the Brazilian strain CPAC 15 (=SEMIA 5079) belonging to the same species (Delamuta et al., 2013); however, the two copies are identical and, therefore, do not invalidate the use of the ITS region. A more defined phylogeny of *Bradyrhizobium* using the ITS genomic region was also obtained in studies performed by our group (Alberton et al., 2006; Germano et al., 2006; Menna et al., 2009a), reinforcing the utility of this locus.

Another advantage of analysis of the ITS relies in a direct relationship with results obtained in the DNA–DNA hybridization (DDH) technique. DDH is still required to define new species (Garrity and Holt, 2001), although an increasing number of arguments against its obligatory use have been raised, including the high cost and intensive

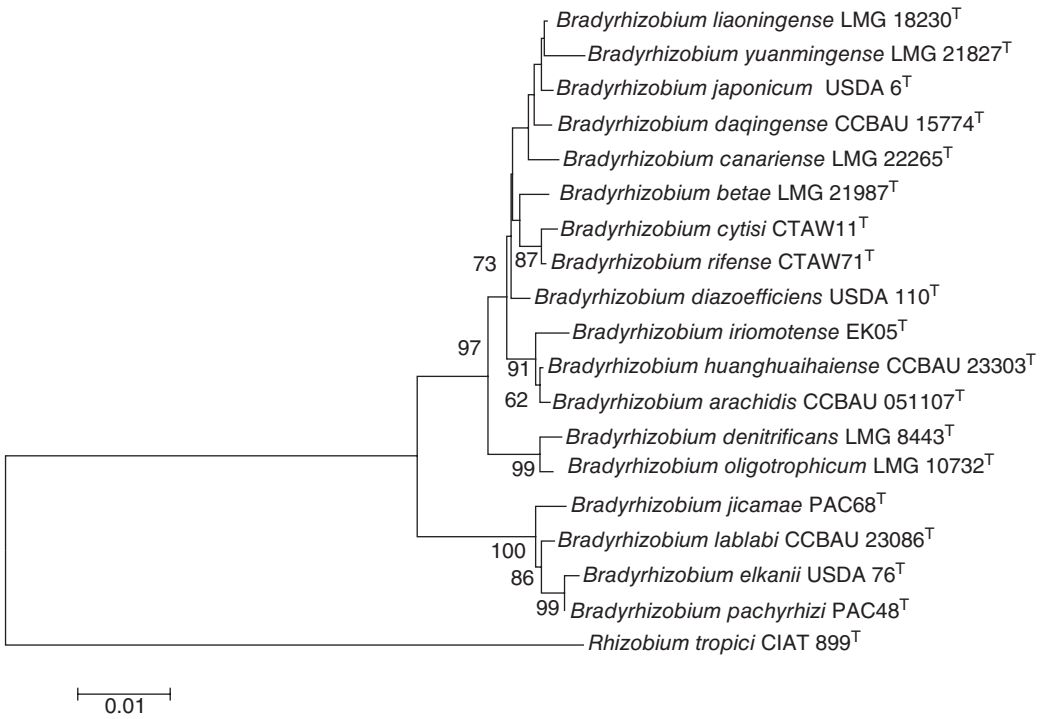

Figure 18.1 Neighbor-joining phylogeny based on 16S rRNA gene sequences showing the relationships between described species of the genus *Bradyrhizobium*. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed using the Tamura–Nei method and are in units of the number of base substitutions per site. Positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted in MEGA5. *Rhizobium tropici* was used as an outgroup. Bar, 1 substitution per 100 nucleotide positions.

work required for its development (e.g., Vandamme et al., 1996; Coenye et al., 2005; Ribeiro et al., 2012), the existence of more accurate approaches (e.g., Konstantinidis and Tiedje, 2004), and doubts about its adequacy (e.g., Achtman and Wagner, 2008). Replacements of DDH have been suggested, such as the use of the average nucleotide identity (ANI) of genome sequences. First, it was proposed that an ANI >94% would correspond to 70% of DDH (Konstantinidis and Tiedje, 2004), and later >95–96% was suggested as the threshold for species delineation (Richter and Rossello-Mora, 2009). With improved facilities available and lower costs of sequencing genomes, replacement of DNA–DNA by ANI is appealing. However, where numerous strains are to be studied, genome analysis is still problematical.

Therefore, when analysis of many genomes is impossible, meaningful results may be achieved from the ITS region, with strong correlations with DDH results (Willems et al., 2001a, 2003). According to Willems et al. (2003), strains of >95.5% similarity in their ITS sequences belong to the same genospecies, showing >60% DNA–DNA hybridization values.

Although 16S rRNA is highly conserved, genetic recombination and horizontal gene transfer may occur within the gene (van Berkum et al., 2003; Gevers et al., 2005). Therefore, the phylogenies of rhizobia based on 16S rRNA, 23S rRNA, or ITS genes may be discordant (van Berkum et al., 2003; Germano et al., 2006). A combined analysis of ribosomal genes was then proposed as a buffer to site-specific horizontal gene transfer, which, at the same time, would be useful to improve species definition, due to the longer sequence of nucleotides, or greater number of bands in RFLP analysis. This polyphasic approach has been used successfully with *Bradyrhizobium* (e.g., Ludwig and Schleifer, 1994; Vinuesa et al., 1998; Germano et al., 2006: Menna et al., 2009a), improving the definition of species.

18.5 THE USE OF THE MULTILOCUS SEQUENCE ANALYSIS (MLSA) APPROACH TO DEFINE TAXONOMY AND PHYLOGENY OF *Bradyrhizobium*

Based on the limitations of the analysis of 16S rRNA, with an emphasis on the lack of capacity of detecting differences among some species – as is the case with *Bradyrhizobium* – other genes with evolution rates faster than that

of 16S rRNA, but conserved enough to retain genetic information, have been proposed as alternative phylogenetic markers (e.g., Stackebrandt et al., 2002; Hernández-Lucas et al., 2004; Parker, 2004; Stepkowski et al., 2003; Martens et al., 2007, 2008; Alexandre et al., 2008; Roma-Neto et al., 2010). Multilocus sequence analysis (MLSA) was then proposed, encompassing the combined analysis of housekeeping genes, and it has been increasingly used to access phylogeny and taxonomy of prokaryotes. Requisites for the choice of the housekeeping genes have been suggested and include the following: (i) broad distribution within the taxa studied; (ii) even distribution in the genome (dispersed in at least 100 kb, to avoid horizontal gene transfer events); and (iii) a single copy within a given genome (Gevers et al., 2005; Martens et al., 2007, 2008). The MLSA has been applied also in studies with rhizobia, including the genera *Sinorhizobium* (e.g., Martens et al., 2007, 2008), *Rhizobium* (e.g., Ribeiro et al., 2009), and *Bradyrhizobium* (Moulin et al., 2004; Vinuesa et al., 2005; Menna et al., 2009a; Delamuta et al., 2013).

Among the housekeeping genes used in the MLSA for phylogenetic reconstructions of rhizobia are the *atpD*, *dnaJ*, *dnaK*, *gap*, *glnA*, *glnII*, *gltA*, *gyrB*, *pnp*, *recA*, *rpoA*, *rpoB,* and *thrC* genes (Turner and Young, 2000; Gaunt et al., 2001; Weir et al., 2004; Stepkowski et al., 2003, 2005; Vinuesa et al., 2005; Martens et al., 2007, 2008; Alexandre et al., 2008; Menna et al., 2009a; Ribeiro et al., 2009, 2012; Delamuta et al., 2013; see Chapter 3).

One question that is often raised concerns the number of genes that should be considered in the MLSA analysis. Martens et al. (2008) found high concordance between MLSA of five housekeeping genes and DNA–DNA hybridization. In *Bradyrhizobium*, the use of at least four housekeeping genes has been proposed (Stepkowski et al., 2003, 2005; Vinuesa et al., 2005); however, more recently, other studies have shown good resolution with only three genes (Menna et al., 2009a; Chahboune et al., 2011, 2012; Chang et al., 2011; Delamuta et al., 2012, 2013; Zhang et al., 2012). One important point to be considered is that the congruence of each gene with the 16S rRNA should be carefully analyzed, eliminating genes with evidence of horizontal gene transfer or atypical evolutionary history. One example was observed in the study by Menna et al. (2009a), in which four housekeeping genes were analyzed, *atpD*, *dnaK*, *glnII*, and *recA*; however, a specific group of *B. elkanii* strains showed high variability in the *atpD* gene, and, thus, the tree built with three genes reflected better the phylogenetic relationships of a large group of *Bradyrhizobium* strains studied than the tree built with all four genes.

For the last decade, we have been working with a large collection of *Bradyrhizobium*, most from Brazil and including about 180 strains isolated from 64 legume species belonging to 17 tribes of the three subfamilies of Leguminosae (Germano et al., 2006; Menna et al., 2009a, 2009b; Binde et al., 2009; Menna and Hungria, 2011; Delamuta et al., 2012, 2013). The results from these studies have clearly shown that the 16S rRNA defines poorly the phylogenetic relationships among *Bradyrhizobium* species. The additional analyses of other ribosomal DNA regions has substantially improved the definition of species (Germano et al., 2006; Menna et al., 2009a) and confirmed the results from Willems et al. (2001a) that ITS is a valuable gene for the taxonomic study of *Bradyrhizobium* (Menna et al., 2009a). MLSA has proven to be extremely valuable for defining taxonomic positions and phylogenetic relationships (Menna et al., 2009a; Delamuta et al., 2012, 2013), and, in Figure 18.2, there is a comparison of results obtained with the analysis of 16S rRNA and MLSA with three genes of a group of *Bradyrhizobium* strains.

18.6 PHYLOGENY OF NODULATION AND NITROGEN-FIXATION GENES IN *Bradyrhizobium*

In our studies with ribosomal and MLSA genes, we have seen poor correlations between taxonomic position and host range (Germano et al., 2006; Menna et al., 2006, 2009a; Binde et al., 2009). For example, several strains were identified that could nodulate and be effective in fixing nitrogen with legumes of different tribes and subfamilies, but there were also more specific groups of strains, such as those symbionts of soybean. To gain a better understanding of nitrogen-fixation ability, we studied *Bradyrhizobium nod* and *nif* genes (see Chapters 3, 8, 20, 77).

It is well known that the symbiosis is under the control of a finely tuned expression of common and host-specific nodulation genes and also of genes related to the assembly and activity of the nitrogenase, which, in the *Bradyrhizobium* strains investigated so far, are mostly clustered in a symbiotic island. It is noteworthy that, in *B. japonicum*, this symbiotic island is flanked by insertion sequence (IS) elements, with high capacity of horizontal gene transfer (Kaluza et al., 1985; Kaneko et al., 2002, 2011). To better understand the evolution of symbiotic genes in *Bradyrhizobium*, we studied 40 strains, symbionts of several legumes; for the nodulation trait we analyzed common [*nodA*], *Bradyrhizobium*-specific [*nodY/K*], and host-specific [*nodZ*] nodulation genes, and also *nifH* for the fixation ability (Menna and Hungria, 2011). One important point is that the chosen genes are distantly located in the symbiosis island of *B. japonicum* (Kaneko *et al*., 2002), avoiding interpretations based on horizontal gene transfer.

In general, clusterings of *nod* and *nif* trees were similar and clearly different from those of other rhizobial species, indicating a monophyletic origin of nodulation and nitrogen-fixation genes in *Bradyrhizobium*; however,

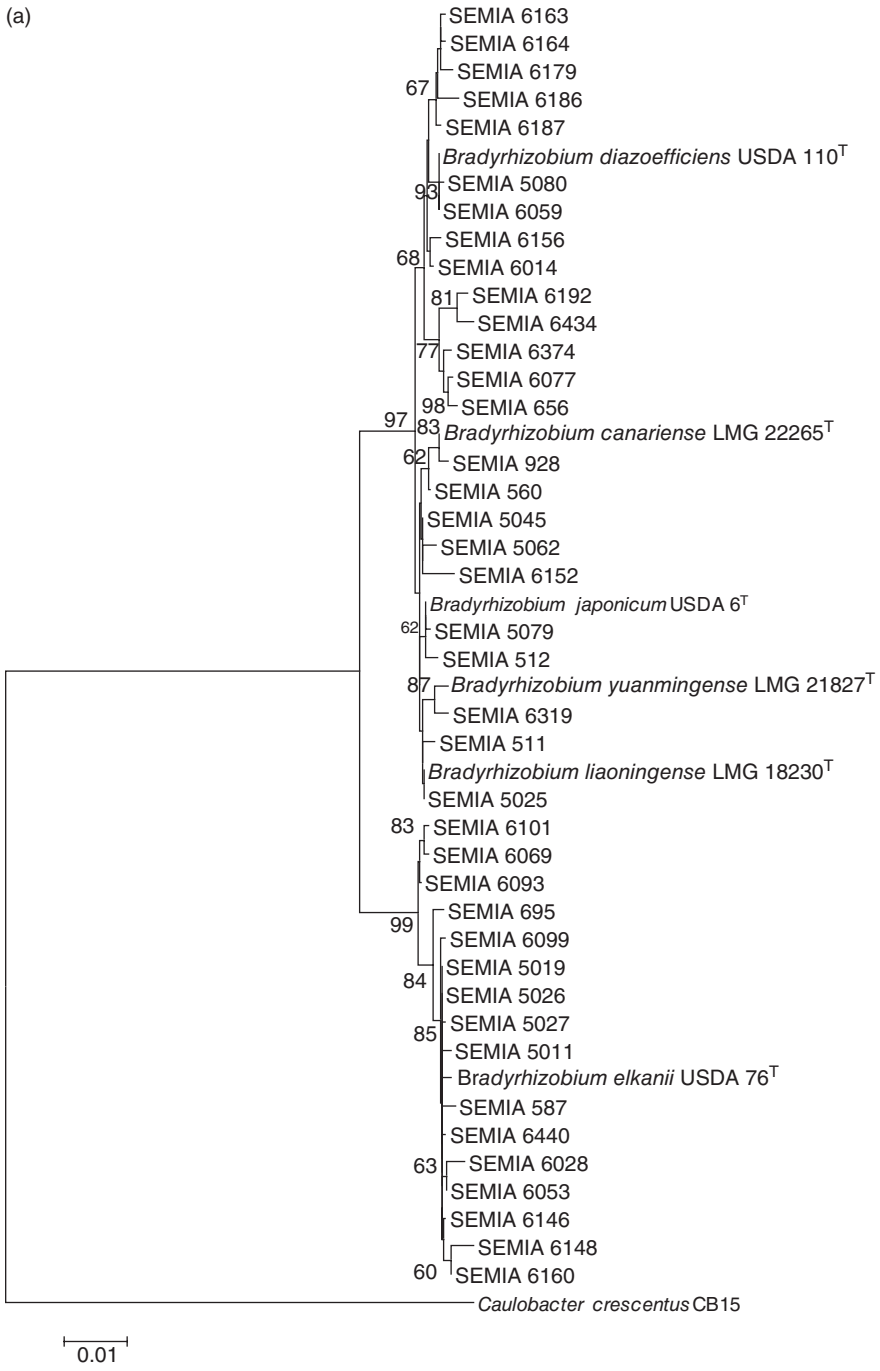

Figure 18.2 Evolutionary trees inferred using the neighbor-joining method for *Bradyrhizobium* strains based on the (a) 16S rRNA gene and (b) concatenated sequences of the *dnaK*, *glnII*, and *recA* genes. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Evolutionary distances were computed using the maximum composite-likelihood method and are in units of the number of base substitutions per site. Positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted in MEGA4. Modified from Menna et al. (2009a).

congruence of these genes with housekeeping genes was low (Menna and Hungria, 2011).

The hypothesis of monophyletic origin of nodulation genes has been raised in studies in which *nodA* of several *Bradyrhizobium* strains fitted into a defined phylogenetic group, in some cases showing correlation with the geographic origin (Stepkowski et al., 2007; Steenkamp et al., 2008; Menna and Hungria, 2011). Figure 18.3 shows the results obtained with strains from our study (Menna and Hungria, 2011). One interesting feature of *nodA* is that it has 209–211 amino acids (Menna and Hungria, 2011), whereas in *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* the gene has 195–198 amino acids (Moulin et al., 2004; Stepkowski et al., 2007). The loss at the N-terminal end of the NodA protein in other rhizobial genera might suggest that the gene is undergoing an evolutionary progression toward a shorter sequence, which also gives support to the theory that *Bradyrhizobium* is the ancestor of all rhizobia.

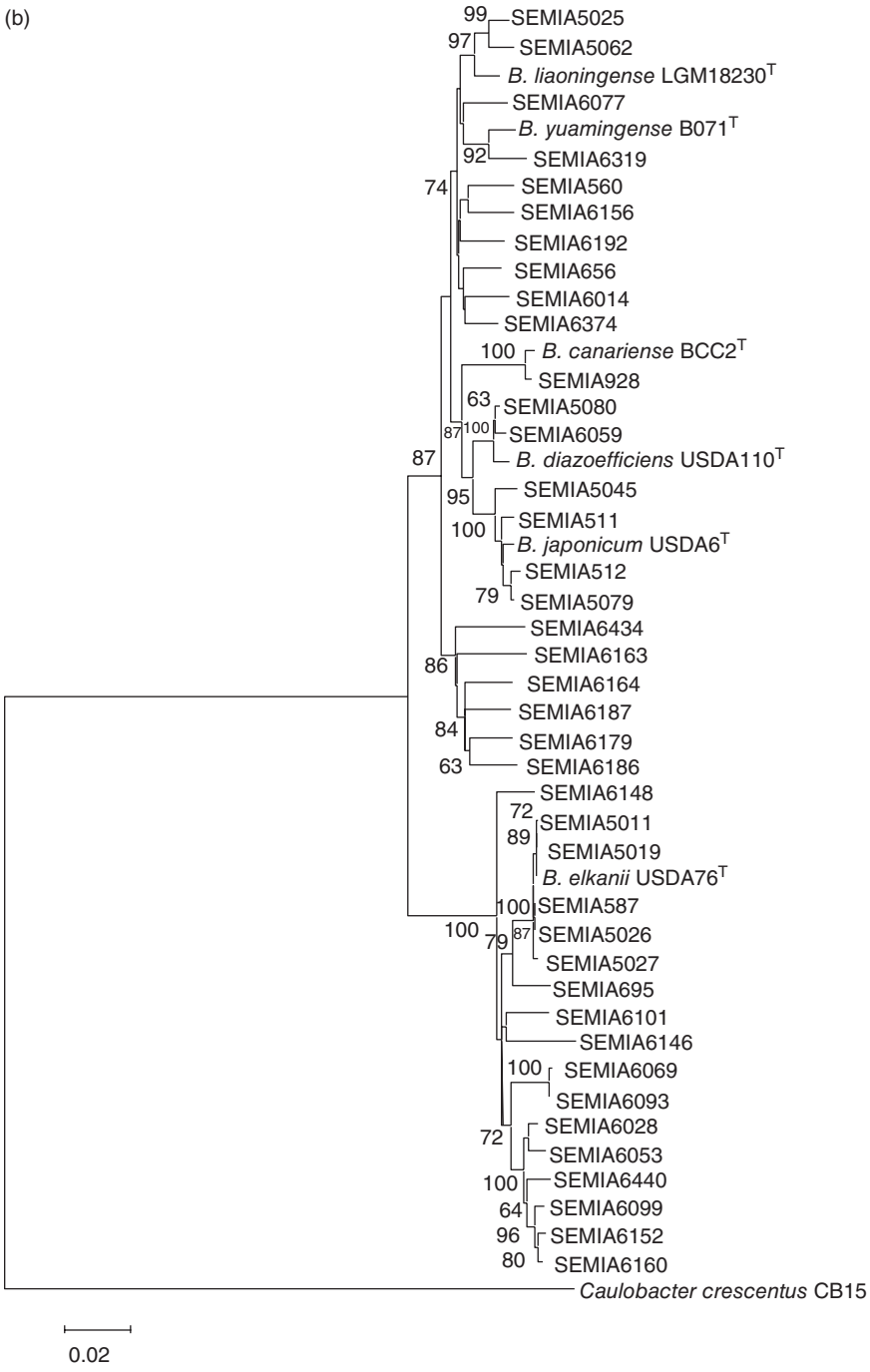

Figure 18.2 (*Continued*)

The results from our study (Menna and Hungria, 2011) have pointed out that *Bradyrhizobium* adopted different nodulation/nitrogen fixation strategies. The first and predominant one included evolution toward increased host range, emphasized by the greater diversity of *nod* genes, with an emphasis on *nodZ*. *nodZ* is unusual in comparison to other common nodulation genes such as *nodABC*, as its expression is constitutive and independent of *nodD*; chemical analysis has suggested that NodZ is essential for fucosylation of the terminal reducing *N*-acetylglucosamine of the lipochitin oligosaccharides (LCOs) of Nod factors (Stacey et al., 1994). The role of host specificity of NodZ has been confirmed in experiments on host range (López-Lara et al., 1996). Two strategies have been detected so far for NodZ. The first and predominant strategy shows high diversity of *nodZ* among *Bradyrhizobium* (Steenkamp et al., 2008; Menna and Hungria, 2011) giving support to the hypothesis that the variability in the decorated nodulation

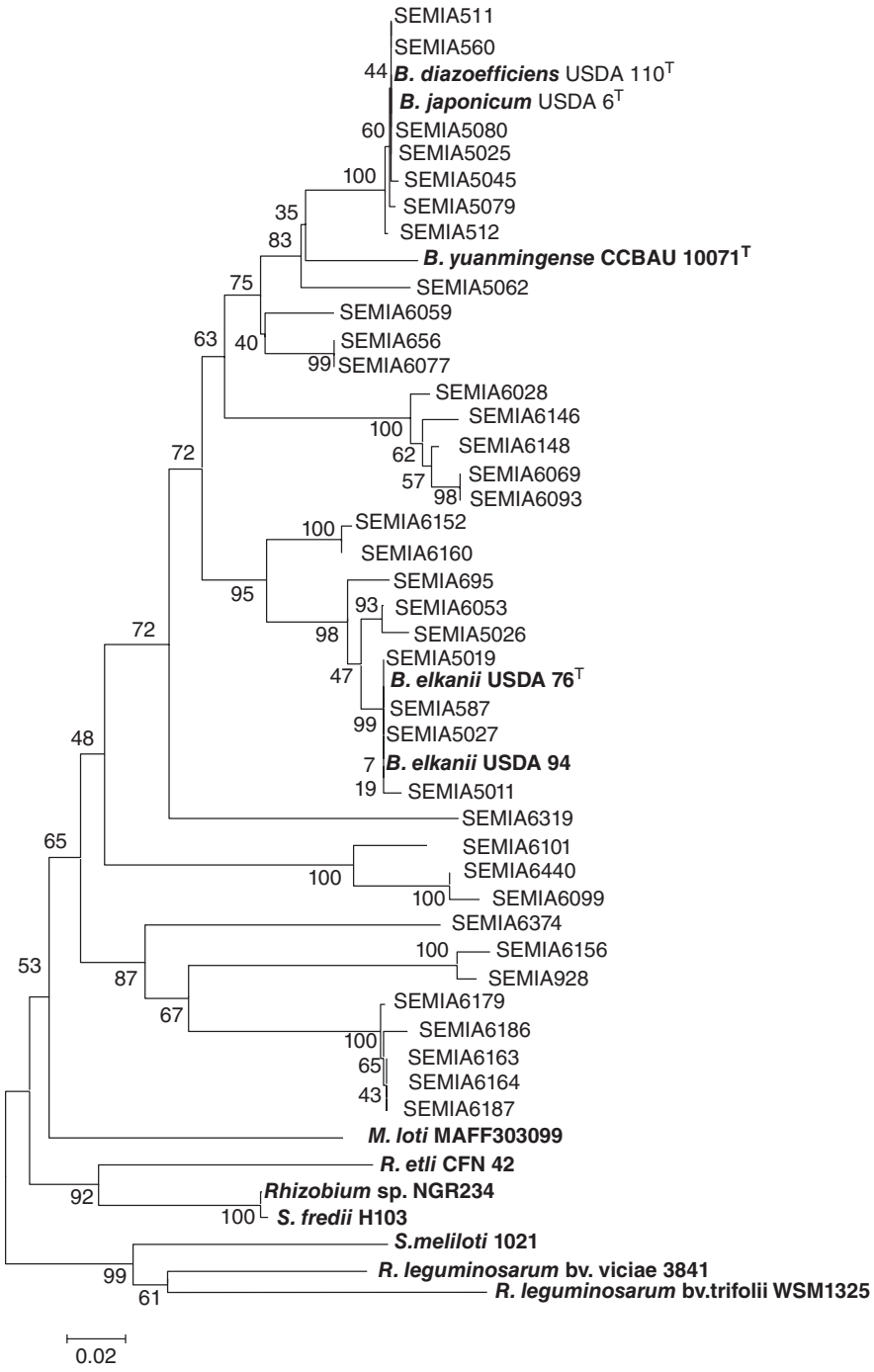

Figure 18.3 Phylogenetic relations of *Bradyrhizobium* SEMIAs and type/reference strains based on *nodA* partial sequences. Type/reference strains are highlighted in bold. Phylogeny was inferred using the neighbor-joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to branches. Evolutionary distances were computed using the maximum composite-likelihood method and are in units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4. After Menna and Hungria (2011).

factors might represent an important adaptation strategy, enabling nodulation of a variety of legumes. The second strategy, less common, shows the absence of *nodZ*, and one example is a group of symbionts of *Acacia* sp. (Menna and Hungria, 2011), which might indicate that a specific fucosylation of the LCOs may not be necessary for nodulation of the host plant, probably due to close coevolution of the symbiotic partners.

However, we identified another group of strains with a different evolution story. A group of symbionts from *A. mearnsii* showed high congruence between the 16S rRNA and the *nodY/K*, *nodA,* and *nifH* genes, in addition to a shorter *nodY/K* and the absence of *nodZ*, strongly indicating a finely-tuned coevolution of the host plant and symbionts. In addition, the *B. japonicum* symbionts from soybean also showed high congruence of *nod* and *nif* genes with the

16S rRNA, albeit to a lesser extent (Menna and Hungria, 2011). Lastly, our study also identified some symbionts of *Arachis* spp. which occupied isolated positions in all *nod* and *nifH* trees, with no relation with the core genes, apparently resulting from horizontal gene transfer.

In conclusion, despite reinforcing the theory of monophyletic origin of the symbiosis island in *Bradyrhizobium*, our study pointed out that horizontal gene transfer is common in a variety of groups, whereas, in others, vertical transfer represents the main genetic event, altogether contributing to a high level of diversity in *Bradyrhizobium* (Menna and Hungria, 2011).

Finally, we must comment that absence of the common *nodABC* genes and of *nodY/K* has been reported for photosynthetic *Bradyrhizobium* strains BTAi1 and ORS278, symbionts from *Aeschynomene* stem nodules (Giraud et al., 2007; see Chapter 28), and other *Bradyrhizobium* strains from our study (Menna and Hungria, 2011). These results indicate that an alternative mechanism for initiating transcription of nodulation genes is not exclusive to the stem-nodulating group, opening an interesting field of research. It is interesting to note that very few *Bradyrhizobium* genomes have been sequenced so far (Kaneko et al., 2002, 2011; Giraud et al., 2007; Tian et al., 2012), and new facilities and lower costs will likely increase our knowledge of the genomics of these strains in the foreseeable future.

18.7 CONCLUDING REMARKS

The family Leguminosae encompasses over 18,000 species, of which about 3000 are capable of nodulating with rhizobia, but information is lacking for nearly 40% of the legume genera. Evidence indicates that *Bradyrhizobium* is the ancestor of all rhizobia, and strains belonging to the genus have been isolated from a variety of legumes and ecosystems, being particularly abundant in the tropics. However, few *Bradyrhizobium* species have been described so far, in part, due to the high conservation of the 16S rRNA gene. The use of other ribosomal and housekeeping genes, with an emphasis on the MLSA method, is facilitating the detection of genetic diversity and the description of new *Bradyrhizobium* species. Analyses of nodulation and nitrogen-fixation genes have shown a predominance of monophyletic evolution of *nod/nif* genes, but with low congruence with the 16S rRNA gene. However, there are also a few groups with specific evolution stories toward higher or lower host specificity. Considering the high number of host legumes in symbioses with *Bradyrhizobium*, very few genetic studies have been performed so far with the genus. Progress is expected in the next few years with the help of the new molecular tools available today, with an emphasis on genomics.

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Chapter 19

Interaction between Host and Rhizobial Strains: Affinities and Coevolution

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19.1 INTRODUCTION

The legume–*Rhizobium* symbiosis is characterized by the formation of a new root organ, the nodule, in which bacteria fix atmospheric dinitrogen and exchange it for photosynthates and other nutrients provided by the host legume. The symbiosis enables legumes to produce protein-rich seed and foliage that are critical to many human and animal diets.

Improvement of crop yield within the context of low-input agriculture is an ongoing concern for breeding programs, in which biological nitrogen fixation can play an important contribution. Nitrogen efficiency for several legumes can vary more than 10-fold providing a potential for optimization. However, while superior N_2-fixing strains have been isolated and tested under controlled environmental conditions, the problem to exploit these strains is the inability to control infection of legume roots in the field by indigenous strains that in many cases induce nodules but fix nitrogen poorly.

The mutual interaction is initiated by the exchange of signals between the plant and the bacteria. A rhizobial lipochito-oligosaccharide molecule called Nod factor, produced in response to (iso)flavonoids exuded by the root, is perceived by two plant LysM receptor kinases. The chemical structure of nodulation factors shares a common backbone of *N*-acetylglucosamine residues, which, in addition, feature diverse substitutions at the reducing and nonreducing ends of the molecule (see Chapters 50 and 51). Perception of Nod factor by the legume host triggers different responses that mediate changes in young epidermal cells and, at the same time, is necessary for the molecular events that will induce cortical cells to reinitiate cell division leading to nodule organogenesis. These responses can occur independently of each other, as it has been shown by the phenotype of different mutants, where bacterial infection – related to the epidermal responses – is observed in the absence of nodule formation and vice versa (Madsen et al., 2010; Oldroyd et al., 2011).

Early nodulation events act to inhibit nodulation of developmentally younger root tissues to maintain a homeostatic balance of shoot and root development. The regulatory circuit is called "autoregulation of nodulation" (AON) (Reid et al., 2013). It has been shown that the AON starts to be active after root hair curling but before the initiation of visible cortical and pericycle cell divisions. Therefore, it is expected that competitiveness for infection will be expressed at very early stages of interaction. Once a certain strain triggers infection, then AON systemic mechanisms may prevent infection by other strains. The legume–rhizobia association is highly specific such that a limited set of rhizobial species

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will establish symbiosis with only a limited number of host plants; however, the mechanisms whereby the host legume controls nodulation by specific strains are poorly understood. This chapter examines the relevant accomplishments to better understand the basis of controlling nodule formation in legumes, and also an attempt has been made to consider how manipulation of the host–bacteria interactions may contribute to overcome the problem of competitiveness for nodulation. The detailed mechanisms involved in the plant response to rhizobial signals are not discussed here, but references can be found in the literature cited.

19.2 Nod FACTOR RECOGNITION

The chemical structure of the rhizobially produced lipochito-oligosaccaride, which is recognized by the host LysM-containing receptor kinases Nfr1 and Nfr5, is a major determinant of host specificity. Thus, the *Sinorhizobium meliloti nodH* mutant that lacks a C-6 sulfate at the reducing end is unable to induce a response on its natural host *Medicago truncatula* (Roche et al., 1991; see Chapter 51).

Radutoiu et al. (2007) have shown that expression of *Lotus japonicus* Nfr1 and Nfr5 in *M. truncatula* and *Lotus filicaulis* extends their host range to include bacterial strains *Mesorhizobium loti* and *R. leguminosarum* bv. viciae strain DZL, which usually only infect *L. japonicus*. They proposed that differences in decorations of the Nod-factors synthesized by *M. loti* and DZL influence affinity at the NFR5 LysM2 domain, which is also associated to a specific amino acid present in this domain.

Two cases that reveal natural variation in nodulation specificity in pea highlight the significance of fitness between the Nod factor decorations and its receptors. The Afghanistan pea cultivar is resistant to nodulation by European *Rhizobium leguminosarum* strains but is efficiently nodulated by strains isolated from Israel. Strains of *R. leguminosarum* bv. viciae that can nodulate cv. Afghanistan produce a Nod factor that has an O-linked acetyl group on the terminal reducing end residue. Acetylation requires a bacterial gene called *nod*X. Further investigations demonstrated that the introduction into the European strains of the ability to acetylate the nodulation factor lifts the restriction and makes them able to nodulate cv. Afghanistan (Firmin et al., 1993). The resistance to nodulation in Afghanistan segregates as a single genetic locus, in a region where there are multiple Nod factor receptor-like genes (Gualtieri et al., 2002).

Another instance of regulation of nodule formation associates the efficiency of nodulation by nodE mutants of *R. leguminosarum* bv. viciae to one haplotype of Sym37 in peas. Sym37 belongs to the family of receptor kinases that includes *L. japonicus* NFR1 and *M. truncatula* LYK3, contains three LysM ectodomains, which in *L. japonicus* have been implicated in binding of Nod factors (Broghammer et al., 2012; Ronghui et al., 2011). *NodE* mutants are unable to form C18:4 acyl chains and, therefore, produce only C18:1-containing Nod factors. Because the six polymorphic residues of Sym37 are spread across the three LysM domains, it is possible that such substitutions may cause important structural changes that affect recognition.

LysM-containing receptor-like kinases (LYKs) are found only in plants including the primitive plant genera *Physcomitrella* and *Selaginella.* It has been proposed that a more recent specialization took place in legumes in order to provide specificity and efficiency to the recognition of rhizobial nodulation signals (Zhang et al., 2009).

19.3 MECHANISMS USING PLANT IMMUNE RESPONSES

Analysis of host genes induced in response to rhizobial infection has revealed that several of the upregulated genes are also genes involved in the mechanisms of plant defense response to pathogens. Peltzer-Meschini et al. (2008) has described genes homologous to soybean Rpg1-b to be highly induced in *Phaseolus vulgaris* at early stages after infection with its cognate *Rhizobium etli*. An interesting case is the pair of genes NSP1 and NSP2 of the group of GRAS transcription factors from *M. truncatula*, which are essential for the Nod factor signaling pathway, whereas their orthologs from rice are induced by perception of a chitooligosaccharide elicitor. The current scenario indicates that legumes activate quite similar mechanisms to respond to pathogens and symbiotic microbes (Kalo et al., 2005; Samac and Graham, 2007), and that in nature legumes genes have been recruited from the plant resistance pathway to control nodulation. This has been clearly documented in soybean in which several genes have been identified to restrict nodulation with specific rhizobial strains. The genetic analysis of the loci that in soybean causes ineffective nodulation has revealed they map in a region of the genome that contain sequences determinant of resistance to pathogenic microorganism, including the genes Rj2 and Rfg1, which restrict nodulation with *Bradyrhizobium japonicum* strain USDA122 and *Sinorhizobium fredi* strain USDA257, respectively (Caldwell, 1966; Trese, 1995). In a recent report by Yang et al. (2010), it was shown that Rj2 and Rfg1 are allelic genes encoding a member of the Toll-interleukin receptor nucleotide binding site/leucine-rich repeat (TIR-NBS-LRR) class of plant resistance proteins. This study indicated that successful nodulation requires rhizobia to evade the plant immune responses triggered by microbial infection, and define a way outside the Nod-factor-mediated mechanism to control nodulation by specific strains.

19.4 THE SYMBIOTIC INTERACTION OF *Phaseolus vulgaris* IN THE REGIONS OF HOST DIVERSIFICATION

Common beans (*P. vulgaris* L.) is one of the most ancient crops in America with a proposed Mesoamerican origin and two major centers of bean genetic diversification (BD), namely the Mesoamerican center (Mexico, Central America, and Colombia) and the Andean center in South America (Ecuador, Perú, and Argentina) (Gepts, 1998; Bitocchi et al., 2012). *P. vulgaris* can be nodulated by a number of diverse rhizobial genotypes, among which *R. etli* bv. phaseoli is the predominant species found associated with common beans from Mexico, Colombia, and the South Andes. The occurrence of host-dependent competitiveness in the interaction between common bean and *R. etli* was shown in our laboratory (Aguilar et al., 2004). Coinoculation of common beans from each BD center with an equicellular mixture of sympatric and allopatric *R. etli* strains representative of the Mesoamerican and Southern Andean lineages revealed a distinct host-dependent competitiveness: beans from the Mesoamerican genetic pool were almost exclusively nodulated by strains from their host region, whereas nodules of beans from the Southern Andes were largely occupied by the geographically cognate *R. etli* lineages. The different strains of *R. etli* from the Mesoamerican and Andean regions could be distinguished on the basis of the types of *nod*C gene they contain, which for convenience have been denominated α and δ, respectively (Fig. 19.1).

Attempts to investigate whether the diversity in nodulation behavior by different genotypes of *R. etli* was due to differences in the chemical structure of the nodulation factor revealed no significant difference between them. Indeed, it was found that in general the length of the glucosamine oligosaccharide backbone was predominantly pentameric. The C6 of the nonreducing end residue was frequently, but not always, O-carbamoylated. Carbamoylation was detected in all the strains we examined as well as in the *R. etli* type strain CFN42. The N-acyl moiety comprises monounsaturated C_{16} or/and C_{18} fatty acids. Linked to the C6 of the reducing terminal glucosamine residue, a 4-O-acetyl fucose residue was found in all the strains but strain 55N1 which in detailed analyses consistently showed an absence of the O-acetyl decoration (Gil-Serrano, Megias, and Aguilar, unpublished). Therefore, no obvious detectable changes in the Nod factor structures associated with either α or δ *R. etli* strains were found in our analysis, which suggested that differences in affinity between genotype of *R. etli* and common beans is not determined by chemical modifications of the Nod factor.

Common bean is able to establish effective symbiosis with a diversity of rhizobial phylotypes that produce different Nod factors. However, there are clear differences among them in their competitiveness for infection. For instance, the *nol*L mutant strain *R. etli* CFN289 – unlike its parental strain CE3 – produces a Nod factor that lacks an acetyl group at the fucosyl residue at the reducing end of the signal molecule (Corvera et al., 1999). The *nol*L mutant strain induces effective nodules although it is less efficient at nodulation of common beans, and it competes very poorly for nodule occupancy as compared with the wild-type strain (Fig. 19.2).

In order to assess if acetylfucosylation of the Nod factor was important for the nodulation preference between lineages of *R. etli* and beans from the same region of diversification, competitiveness assays were performed with a mixture of strains *nodC* type α and type δ strains that produce Nod factor with and without the acetyl-group at the fucosyl site. The *R. etli nol*L mutant strain (acetyl-minus) was found to be less competitive in comparison with its parental strain CE3 as well as with the *nod*C type α strain SC15 (Fig. 19.2). In case of a mixture of strains α (CE3) and δ (136N2), respectively, that are able to acetyl-decorate their Nod factor at the reducing terminal, the occupancy of nodules was found to be as expected: the strain α CE3 was highly competitive against strain delta 136N2 in the Mesoamerican bean variety cultivar Nag12. In contrast, the *nol*L strain displayed a significantly impaired competitive ability, only 22% of nodules were occupied by CFNX289 (Fig. 19.2). This suggests that the α strain CFNX289, which produces Nod factor without acetylation, is unable to compete and occupy nodules as efficiently as the δ strain 136N2, which produces acetylated Nod factor. However, when competitiveness was assessed between two strains α (CFNX259) and δ (55N1), both of them unable to produce acetylated Nod factor, the strain CFNX259 displayed a clear competitive ability (Fig. 19.2). Taken together, these results suggest that the acetylfucose substitution is involved in the nodulation competitiveness and that the high competitiveness observed with the *nol*L strain CFNX259 against the strain 55N1 suggests that factors other than acetylfucosylation may be also involved in the nodulation preference between host and rhizobia from the same center of genetic diversification.

Common beans have been considered a poor nitrogen-fixing legume, and attempts to introduce superior common bean nodulating strains by inoculation faced the problem how to control infection in the field, since the resulting nodules were mostly formed by highly competitive, but less efficient, indigenous strains. Apparent competitiveness of rhizobia may vary depending on the plant genotype used as a host, which suggests the occurrence of plant genotype × rhizobial strain effects.

Our results are consistent with a competitiveness that is expressed at early stages of interaction, in which the decoration of the nodulation factor is important as well as other factors that affect competitiveness. These results suggest the occurrence of checkpoints other than the nodulation factor in the establishment of preferential nodulation of Mesoamerican beans by cognate *R. etli* strains.

The molecular basis of mutual affinity between common beans and rhizobia from the same center of diversification

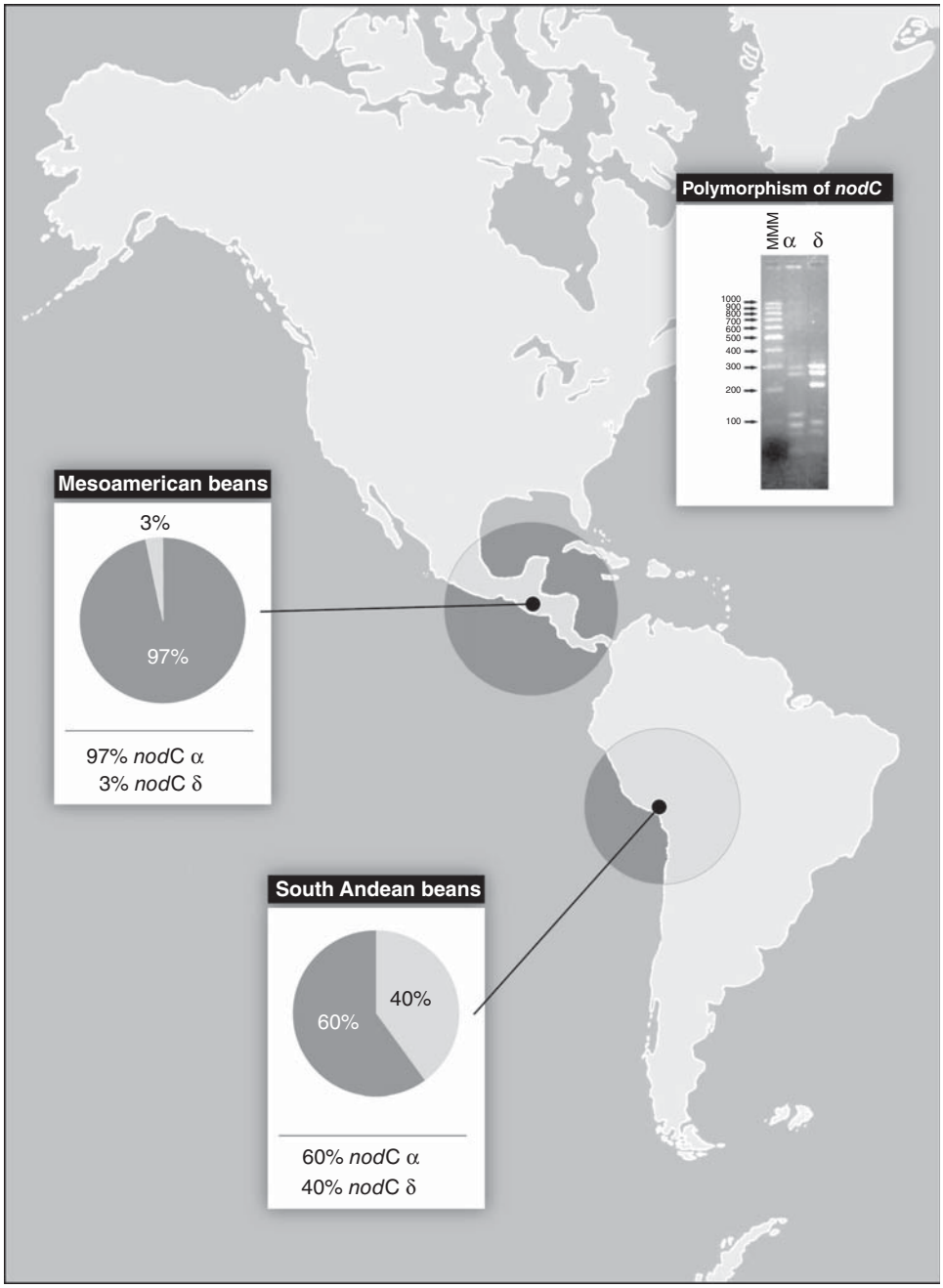

Figure 19.1 Affinity of common beans from the Mesoamerican and South Andean centers of diversification and *R. etli*. Lineages of *R. etli* from Mesoamerica and South Andes regions were distinguished by their *nod*C polymorphism and identified as *nod*Cα and *nod*Cδ, respectively. Nodule occupancy by *nod*Cα and *nod*Cδ strains in coinoculation assays of common beans from the Mesoamerica and South Andean regions is shown as a "cake" graph.

is still unknown. In order to gain insight into the strain preference by beans belonging to the Mesoamerican pool, a survey of genes was performed by applying a suppressive hybridization approach in which a Mesoamerican variety was infected with *R. etli* strains from the Andean and Mesoamerican regions, respectively (Peltzer-Meschini et al., 2008). Among the genes differentially expressed, NF-YC1 encoding for a protein highly homologous to the C subunit of the NF-Y heterotrimeric transcription factor was detected (Zanetti et al., 2010; Battaglia et al., 2014). A relative early accumulation of NF-YC1 transcripts in Mesoamerican beans in response to *R. etli nod*Cα strains was found. Overexpression of NF-YC1 caused no significant change in the preferential pattern of nodulation, whereas it

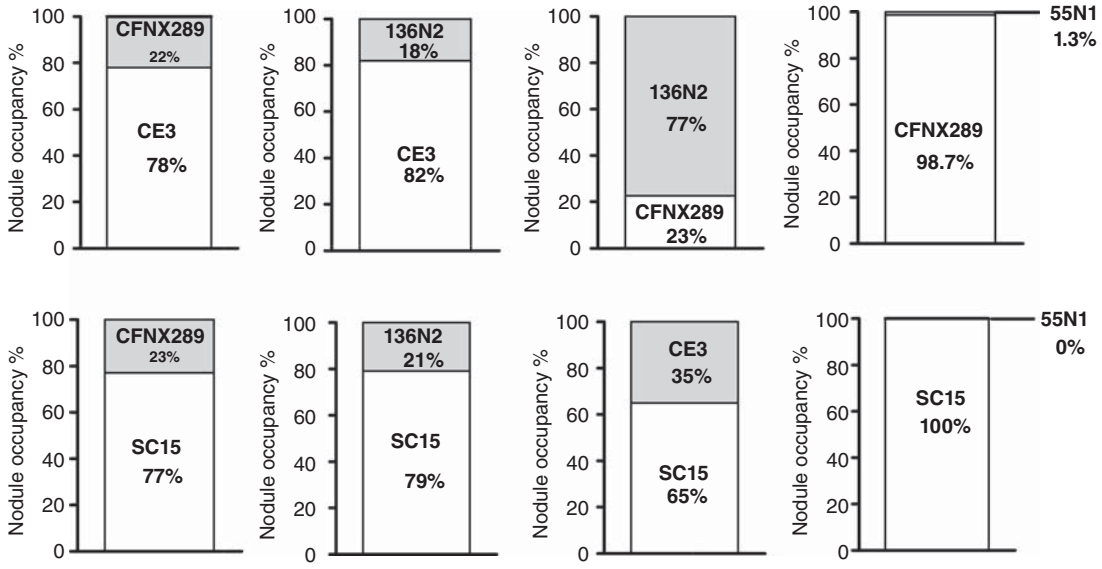

Figure 19.2 Nodule occupancy in the Mesoamerican bean variety cv. Nag12 infected with mixed inoculums of *R. etli* strains. Strains CE3, CFNX289, and SC15 are lineages *nod*Cα, whereas strains 55N1 and 136N2 are *nod*Cδ. Strain CFNX289, which is an *in vitro* induced *nol*L mutant derived from CE3 and strain 55N1, which is an isolate from the South Andean region carrying an intrinsic *nol*L mutation, produce Nod factor without acetylation of the fucosyl residue at the reducing end of the lipochito-oligosaccharide molecule. Bars represent percentage of nodule occupation after inoculation with a two-strain mixture.

promoted the formation of higher number of nodules with the nodCδ strains. Because Zanetti et al. found a relationship between expression of NF-YC1 and genes participating in the G2/M transition of the cell cycle, they suggested that NF-YC1 promotes earlier nodulation by the activation of cortical cell division. Therefore, these results indicate that the occurrence of functional compatibility, probably recruited during the coevolution of both symbionts, leads the interaction to proceed faster toward infection and nodule occupation as compared with the association with allopatric strains. This kind of mechanism defines a natural strategy that the host established in order to select its partner.

19.5 THE NEED OF FACTORS OTHER Nod FACTOR FOR SPECIFICITY IN NODULATION

Studies on the symbiosis between species of Lotus and rhizobia seem to indicate that other factors in addition to nodulation factor are required for efficient recognition and nodulation. *L. japonicus* and *Lotus pedunculatus* produce effective nodules when infected by *M. loti* and *Bradyrhizobium* sp. strains, respectively. In cross-infection assays, it was shown that both of them pass the epidermal stage of recognition. Analysis of the chemical structure of nodulation factors produced by *M. loti* and *Bradyrhizobium* revealed differences among them, particularly decorations at the nonreducing end of the signal molecule. Similarly, differences in the extracellular LysM domain of Nfr1 and Nfr5 from *L. japonicus* and *L. pedunculatus* were shown by gene sequencing. Interestingly, genetic transformation by using *Agrobacterium rhizogenes* and chimera receptors as well as wild-type receptors resulted in no change of the infection pattern in *Lotus* species. These observations led the authors to conclude that an additional checkpoint determine successful nodulation (Bek et al., 2010). Although the functions of other components in the microsymbiont remain to be determined, it should be kept in mind that in addition to nodulation factors, surface rhizobial compounds such as exo- and lipooligosaccarides are also important in nodulation (see Chapter 36).

19.6 CONCLUSIONS

In order to improve nitrogen fixation, it is desirable that at early stages, the legume plant selects a rhizobia partner such that later on their association – directed by bacterially produced signal molecules – results in the formation of the nodule able to efficiency fix and deliver nitrogen. It has been shown that in natural soils, high doses of inoculants with selected strains are needed in order to displace the indigenous, less efficient, strains (Streeter, 1994; Aguilar et al., 2001); however, to achieve high level of inoculums is not practical. The process of infection displays sophistication and selectivity in favor of a single rhizobial species, but it cannot ensure the exclusion of ineffective variants that will be of no benefit for the host: once established inside the nodule, poor or ineffective rhizobia may be sanctioned by the plant (Kiers and Denison, 2010).

The perception of the nodulation factor by plant receptors opens biotechnological possibilities to target and modify a key event in the control of nodulation by specific strains,

whereas the notion that soybean plays control of nodulation by using plant resistance genes suggests yet another strategy to be exploited. The compatibility that became established between host and rhizobia through coevolution in the same region of differentiation must also be taken in consideration. The affinity detected in common beans from the two major center of diversification toward their sympatric *R. etli* strains opens new avenues to identify allelic variations in the host that contribute to such compatibility. Progress in the sequencing of the genomes of beans from both center of domestication, which is close to completion, will allow comparisons and therefore identification of candidate genes likely involved in this mutual affinity. These studies may lead to define genes and genetic markers useful in breeding programs for improved nitrogen fixation.

Rhizobia in the rhizosphere of legume are influenced by exudate compounds, some of which may act as chemoattractants (Cooper, 2007). Therefore, a combination of a legume able to produce and exude a certain compound and an elite strain able to interact positively with the latter could improve competitiveness and suppress infection by low-performing strains.

Overall, it is tempting to speculate that the application of the significant progresses made in our knowledge of the symbiosis will lead to have customized varieties that express interaction with elite rhizobial strains in the future.

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Chapter 20

Assessment of Nitrogenase Diversity in the Environment

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20.1 INTRODUCTION

Biological nitrogen fixation emerged early in the history of life as a fundamental link in the global nitrogen cycle. This process is carried out by diverse diazotrophic microorganisms found exclusively in the domains Bacteria and Archaea. Although most described diazotrophs are found in the Proteobacteria, nitrogen fixation is widespread among prokaryotes and is found in at least 14 bacterial and archaeal phyla (Dos Santos et al., 2012; see Chapter 3). Described species of diazotrophs conserve energy through a wide variety of mechanisms and they can be heterotrophs, lithotrophs, or phototrophs. Diazotrophic species can also have diverse relationships with oxygen, and both obligate and facultative aerobes as well as many anaerobes have been described. In addition, diazotrophs can vary dramatically in their ecology, occurring as free-living organisms in a wide range of environments and also participating in a diverse array of symbiotic associations.

The nitrogenase enzyme catalyzes the conversion of dinitrogen gas to ammonium and comprises protein subunits encoded by the genes *nifH*, *nifD*, and *nifK*. The genes *nifD* and *nifK* encode proteins, which form the heterotetrameric core of the enzyme, also called the MoFe protein because of the requirement for a MoFe cofactor. The *nifH* gene encodes the dinitrogenase reductase subunit, also called the Fe protein. Of these three genes, *nifH* is the most widely sequenced and has become the marker gene of choice for studying the ecology and diversity of nitrogen-fixing organisms (Zehr et al., 2003; Gaby and Buckley, 2011). A typical approach used to explore diazotroph diversity is to generate *nifH* amplicons from environmental samples by using polymerase chain reaction (PCR) with primers that target the *nifH* gene (Zehr and McReynolds, 1989; Marusina et al., 2001; Poly et al., 2001; Ando et al., 2006). The sequences of these *nifH* amplicons can then be analyzed to explore the phylogeny and diversity of diazotrophs present.

The phylogeny of the *nifH* gene and its paralogs may be divided into five principal clusters. According to the designation of Chien and Zinder (1994), conventional nitrogenases fall into two clusters. Cluster I contains primarily aerobes and facultative anaerobes, many of which are found in the phyla Proteobacteria and Cyanobacteria. The second cluster of conventional nitrogenases, cluster III, contains strict anaerobes from diverse phyla and includes methanogenic Archaea, clostridia, spirochetes, and sulfur- and sulfate-reducing Deltaproteobacteria. Cluster II contains alternative nitrogenases, paralogs of *nifH*, which differ from conventional nitrogenase because they use different metal cofactors. There are two alternative nitrogenases, one of which has an FeFe cofactor, whereas the other has a VFe cofactor (see Chapter 2). The FeFe nitrogenase forms cluster II, whereas the VFe *nifH* does not form a coherent cluster, associating mostly with cluster I sequences in phylogenetic trees. Cluster IV contains *nifH* paralogs present in methanogens, and these genes have not been shown to function in nitrogen fixation (Staples et al., 2007). Cluster V contains *nifH* paralogs involved in bacteriochlorophyll synthesis (Raymond et al., 2004).

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Awareness of the presence of these paralogs is essential when working with PCR primers for *nifH* because paralogous gene sequences can be amplified by many *nifH* primer sets (Ohkuma et al., 1999; Yamada et al., 2007) and can be confused with *nifH* if phylogenetic analysis is not conducted to identify and remove these paralogs prior to analysis of *nifH* diversity.

This chapter provides a summary of our current understanding of the global diversity of nitrogen fixers. It is clear that we still have much to learn about diazotroph diversity and ecology, but advances in technology are contributing to a rapid pace of new discoveries. Next-generation sequencing technologies can now be applied to surveys of *nifH* diversity (e.g., Farnelid et al., 2011, 2013; Mao et al., 2011), and quantitative PCR assays have been developed to assess *nifH* gene abundance in the environment (e.g., Church et al., 2005; Wallenstein and Vilgalys, 2005; Hayden et al., 2010). Application of these new tools is facilitated by the availability of a *nifH* sequence database.

20.2 DATABASES AVAILABLE FOR *nifH*

The availability of an aligned *nifH* sequence database facilitates the analysis of diazotroph diversity and phylogeny as well as the evaluation and design of PCR primers that target *nifH*. There are three publicly available collections of aligned *nifH* sequences. The first is available from the Functional Gene Pipeline/Repository, known as FunGene (Cole et al., 2009). The sequences in FunGene are pulled from public databases and aligned using HMMer. The FunGene sequence compilation is available in FASTA format, but this collection lacks sequence-associated metadata. A second *nifH* sequence collection is available from the Zehr research group (http://pmc.ucsc.edu/~wwwzehr/research/database/) in ARB database format. This database is compiled using a set of representative NifH protein sequences using BLAST to identify NifH in public repositories. A third *nifH* database was created by the authors of this chapter (Gaby and Buckley, 2011, 2012, 2014). This third database was also created in the ARB environment (Ludwig et al., 2004), which allows a user to access sequences and the associated metadata through a graphical user interface. In addition to *nifH* sequences, this database includes linked *nifH*, *nifD*, *nifK*, and 16S rRNA gene records from sequenced genomes. The database includes a comprehensive annotated phylogenetic tree of all *nifH* sequences and trees of the full-length sequences for *nifH*, *nifD*, *nifK*, and 16S rRNA genes. These trees can be used to organize and navigate sequence data. In the following sections, we will explore the diversity of the 32954 *nifH* sequences available in this *nifH* database (http://www.css.cornell.edu/faculty/buckley/nifH_database_2012.arb) as of May 16, 2012.

20.3 DESCRIPTION OF THE *nifH* DATABASE

The *nifH* gene is one of the most frequently sequenced of microbial functional genes (Fig. 20.1). The number of *nifH* genes available in public sequence databases has increased dramatically over time and is continuing to accelerate with the development of next-generation sequencing platforms. In fact, more than 90% of *nifH* sequences in the database have been deposited since 2005 (Fig. 20.2). Sequences in the database are derived from 1211 studies each contributing between 1 and 1299 sequences. There are 584 studies that contributed only 1 sequence (48% of the total number of

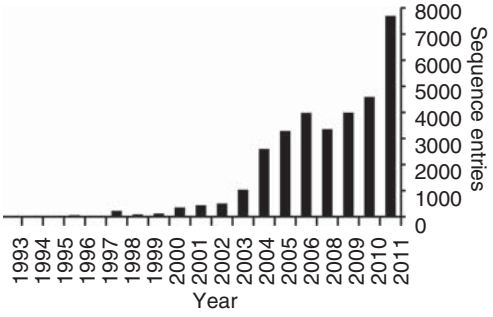

Figure 20.2 The number of *nifH* sequences submitted yearly to GenBank.

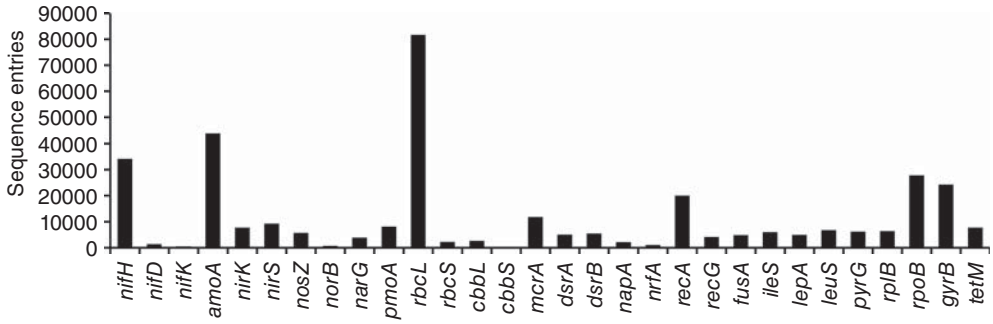

Figure 20.1 The number of sequences available in GenBank for highly sequenced functional genes as of July 12, 2012.

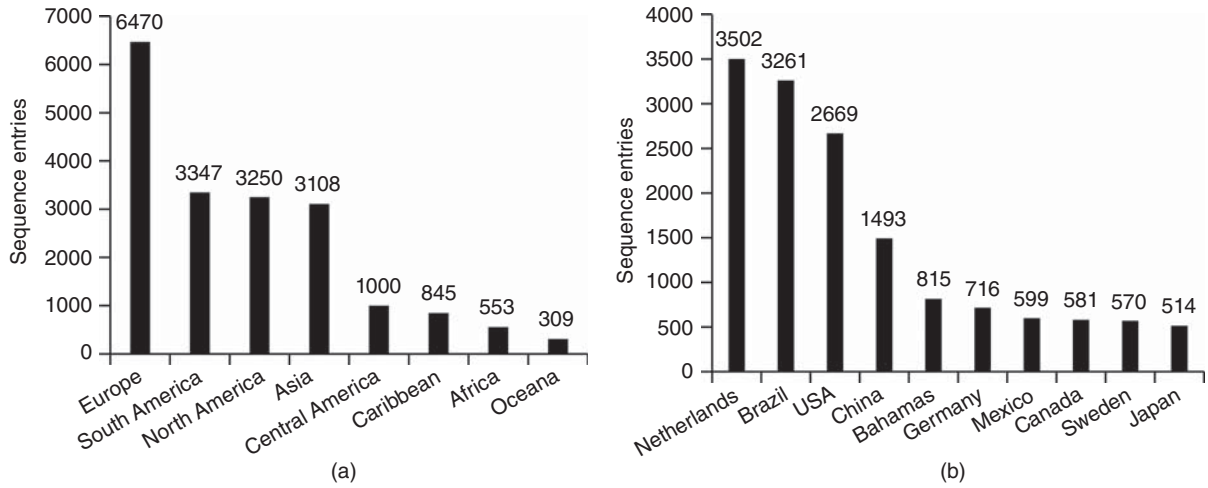

Figure 20.3 The number of *nifH* sequences obtained from the most frequently sampled world regions (a) and countries (b).

studies and 1.8% of the sequences in the database), and these represent sequences that originate from isolated strains. In contrast, the 10 studies that contributed the most sequences account for 8516 sequences in total (26% of all sequences in the database) and the top 35 studies contributed 50% of all sequences.

Evaluation of sequence-associated metadata reveals that, while a vast diversity of nitrogen-fixing microorganisms have been identified through analysis of *nifH*, global sampling efforts have been geographically biased. The *nifH* data disproportionately represent North America and Europe and underrepresent the Southern Hemisphere (Fig. 20.3). It is noteworthy that more than two-thirds of the *nifH* sequences from South America come from a single Brazilian study (Fig. 20.3b). The majority of *nifH* sequences that are available have been generated by a relatively small number of research groups.

20.4 DEFINING *nifH* DIVERSITY

In order to estimate the global richness of nitrogen fixers, it is necessary to establish units for measuring diversity. The conventional approach is to establish an operational taxonomic unit (OTU) based on a defined threshold of nucleotide sequence similarity.

Often an OTU similarity threshold is selected to correspond to a level of sequence similarity that effectively delimits microbial species. In the case of 16S rRNA gene sequences, the most common OTU definition is based on 97% sequence similarity (Stackebrandt and Goebel, 1994). In contrast, a 95% sequence similarity threshold has been proposed as the species-level cutoff for most conserved, protein-encoding genes (Konstantinidis and Tiedje, 2005).

One strategy for selecting an *nifH* OTU definition is to select an *nifH* sequence similarity threshold that roughly

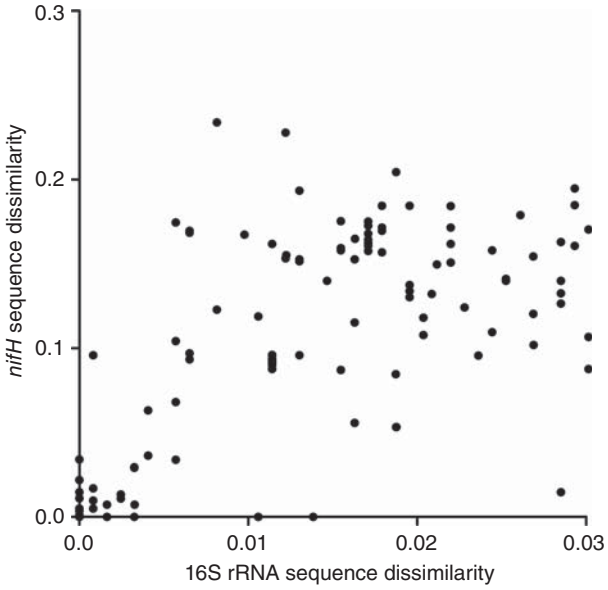

Figure 20.4 The similarity of *nifH* gene sequences for pairs of strains that have less than 3% 16S rRNA sequence similarity. Sequence dissimilarities are calculated from the pairwise comparison of nucleotide sequence divergence for both the *nifH* and 16S rRNA genes from sequenced genomes.

corresponds with the 16S rRNA OTU definition. Pairwise analysis of full-length *nifH* and 16S rRNA genes from sequenced genomes reveals that this approach is somewhat problematic (Fig. 20.4). Strains that fall into the same $OTU_{0.03}$ as defined by 16S rRNA dissimilarity can have *nifH* genes with as much as 23% 16S rRNA sequence dissimilarity (Fig. 20.4); hence, any *nifH* OTU definition is unlikely to correspond closely with species as defined by the 16S rRNA gene. Nonetheless, in order to estimate the diversity of nitrogen-fixing taxa, which have been sampled

globally, and to determine the extent to which sampling efforts have recovered extant nitrogen fixer diversity, it is necessary to establish an OTU definition that can capture meaningful units of diversity even if these units do not correspond with microbial species. From these results, a 10% dissimilarity cutoff ($OTU_{0.10}$) was arbitrarily selected as an appropriate operational taxonomic cutoff to define *nifH* sequence diversity, although in most cases it will be more informative to evaluate sequence diversity across several sequence similarity thresholds.

20.5 PHYLOGENETIC DIVERSITY

Nitrogenase *nifH* sequences characterized to date are not distributed evenly across the various clusters and subclusters that comprise the *nifH* phylogeny (Fig. 20.5). Cluster I *nifH* sequences are most common in the *nifH* database representing 19365 or 59% of available sequences. Most of the cluster I sequences (10653 sequences) belong to members of the Alpha-, Beta-, and Gammaproteobacteria, and these fall primarily into subclusters 1J and 1K. Another large group (4530 sequences) of cluster I *nifH* sequences belong to the Cyanobacteria, which are found exclusively in subcluster 1B. The final major group (1925 sequences) of cluster I *nifH* sequences comprises Deltaproteobacteria of the Desulfuromonadales, which form subcluster 1A (Fig. 20.5). In comparison, cluster III sequences are less abundant in the database (3461 sequences). This is likely due to the fact that anoxic environments, which tend to contain ample cluster III nitrogenase sequences, have been sampled for *nifH* far less frequently than arable soils and oxygenated water samples in which cluster I sequences are seen to dominate (Table 20.1).

There were 10833 sequences that shared the same alignment frame (*Azotobacter vinelandii* positions 133–454), and these sequences were used to estimate *nifH* sequence diversity (Fig. 20.6). This set contains a total of 8193 unique *nifH* sequences that cluster into 2341 $OTU_{0.10}$ and 809 $OTU_{0.20}$. Considerable differences can be observed in the richness of the *nifH* clusters and subclusters (Fig. 20.7). At $OTU_{0.10}$, there are significant differences in Chao1 richness estimates between Cyanobacteria (216, 195 LCI, lower 95% confidence interval; 258 HCI, higher 95% confidence interval) and the Alpha-, Beta-, and Gammaproteobacteria (919, 806 LCI, 1076 HCI) and cluster III (2226, 1952 LCI, 2573 HCI) when controlling for sampling intensity (2089th sample; Fig. 20.7a). Cluster III has by far the greatest richness despite having been sampled at half the depth of the Alpha-, Beta-, and Gammaproteobacteria (Fig. 20.7a). In fact, when $OTU_{0.20}$ is used to evaluate the number of deep evolutionary lineages, the difference in richness between cluster III (639, 596 LCI, 702 HCI) and the Alpha-, Beta-, and Gammaproteobacteria (154, 138 LCI, 195 HCI) and the Cyanobacteria (47, 44 LCI, 66 HCI; Fig. 20.7) is even greater (Fig. 20.7b). Cluster III is composed of anaerobic nitrogen fixers (Zehr et al., 2003), and in general anoxic environments such as wetlands and sediments are undersampled relative to oxic environments (Table 20.1). Studies on the evolution of nitrogen fixation support the emergence of nitrogenase in the methanogenic Archaea with subsequent horizontal gene transfer to Bacteria (Boyd et al., 2011). Hence, it is likely that nitrogen fixation originally emerged among strict anaerobes, and this may explain the degree of evolutionary diversity present in cluster III relative to cluster I.

20.6 ENVIRONMENTAL DIVERSITY

Given that habitat sampling has been uneven (Table 20.1), the richness of *nifH* associated with three well-sampled habitats (soils, the marine water column, and microbial mats) was evaluated. To perform the assessment, the Chao1 richness index was calculated with the program DOTUR (Schloss and Handelsman, 2005) using sequence data available in the *nifH* database. Habitats were established by text searches of the sequence record metadata. The results of the assessment show that soil harbors a greater diversity of nitrogen fixers than the photic zone of the ocean (Fig. 20.8). When 2855 sequences are compared to achieve even sampling intensity, the Chao1 richness estimate at $OTU_{0.10}$ for soil (1671, 1496 LCI, 1897 HCI) was significantly higher than for the marine water column (1042, 901 LCI, 1237 HCI). In the case of microbial mats, at a sampling intensity of 1222 sequences, the Chao1 richness estimate for mats (702, 575 LCI, 894 HCI) was not significantly different from the marine water column (760, 608 LCI, 991 HCI), but both mats and the marine water column were significantly lower than soil (1193, 1016 LCI, 1435 HCI; Fig. 20.8).

Diazotroph diversity in marine systems differs dramatically from soils and not just in the total richness of the community as described earlier. The *nifH* sequences from marine samples also display a striking pattern of dominance with a few OTUs from Cyanobacteria present at very high abundance (Gaby and Buckley, 2011). In fact, 5 of the 10 most abundant *nifH* $OTU_{0.10}$ in the database belong to marine Cyanobacteria including species related to *Trichodesmium* and UCYN-A. These five cyanobacterial OTUs alone account for approximately 37% of the marine *nifH* sequences that were used in diversity estimates (i.e., having an alignment frame that overlaps positions 133–454). In contrast, soils and microbial mat communities have few dominant diazotrophs. Soils most frequently contain diverse OTUs belonging to Proteobacteria (subclusters 1J, 1K, and 1A) while microbial mats contain a wide diversity of OTUs from cluster III and Cyanobacteria (Gaby and Buckley, 2011).

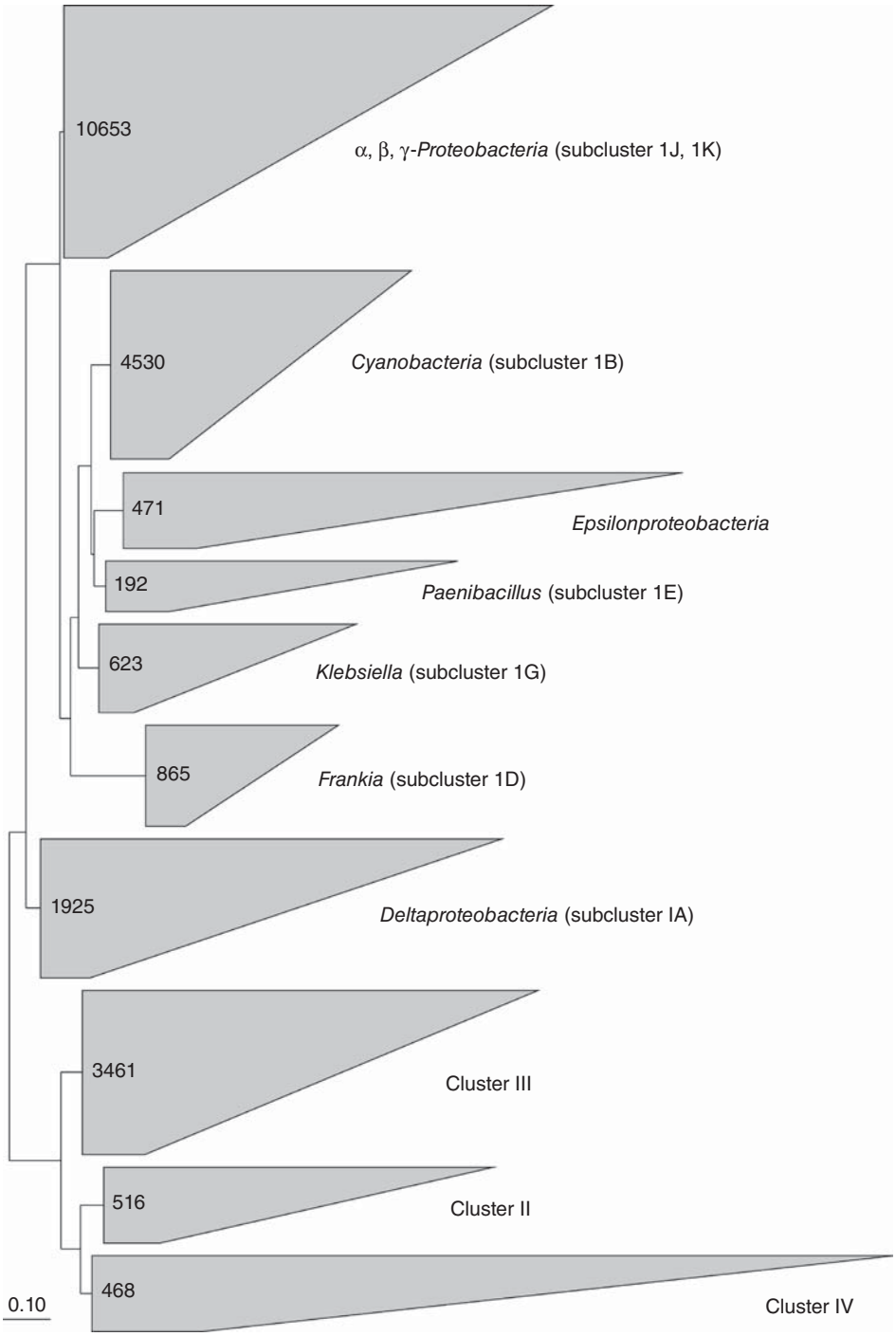

Figure 20.5 Phylogenetic tree showing the major clusters and subclusters of *nifH* sequences. The tree was constructed using a neighbor-joining tree as the base to which most sequences were added using the quick-add-by-parsimony function of ARB. The numbers within the trapezoids are the number of sequences within that phylogenetic group. The selection of groups was informed by groupings formed at an $OTU_{0.40}$ cutoff.

20.7 *nifH* PRIMER ASSESSMENT

Our ability to assess diazotroph diversity is influenced strongly by the availability and efficacy of *nifH* PCR primers. While nitrogenase genes are frequently recovered in metagenomic surveys, diazotrophs are often present as minority populations in microbial communities and so *nifH* genes are often undersampled in approaches that

Table 20.1 The number of *nifH* sequences associated with different environments

Environment	Sequence Number
Terrestrial	17,340
Soil	8,568
Agricultural	7,308
Marine	5,958
Microbial mat	5,141
Maize	3,082
Forest	2,534
Endophytic	1,645
Plant stem	1,407
Salt marsh	866
Rice	854
Termite	590
Lake	430
Grass	416
Thermal	225
Mine spoils	156
Wastewater treatment	69
Soil crust	60

Categories may overlap.

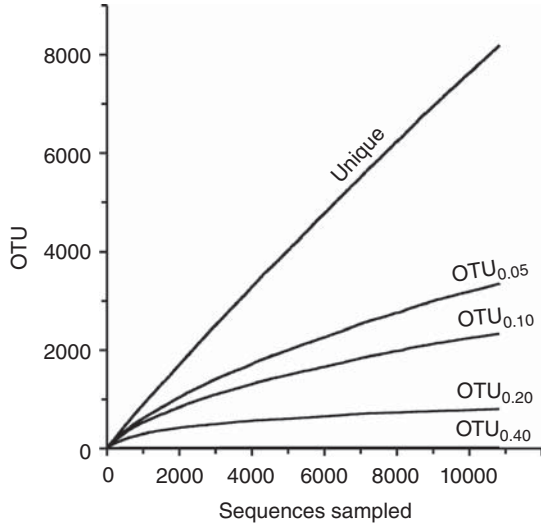

Figure 20.6 Chao1 richness estimates with 95% confidence intervals for all 10833 *nifH* sequences that share an alignment frame spanning positions 133–454 (*Azotobacter vinelandii* numbering). Each line depicts a different sequence dissimilarity OTU clustering threshold as indicated by labeling in subscript.

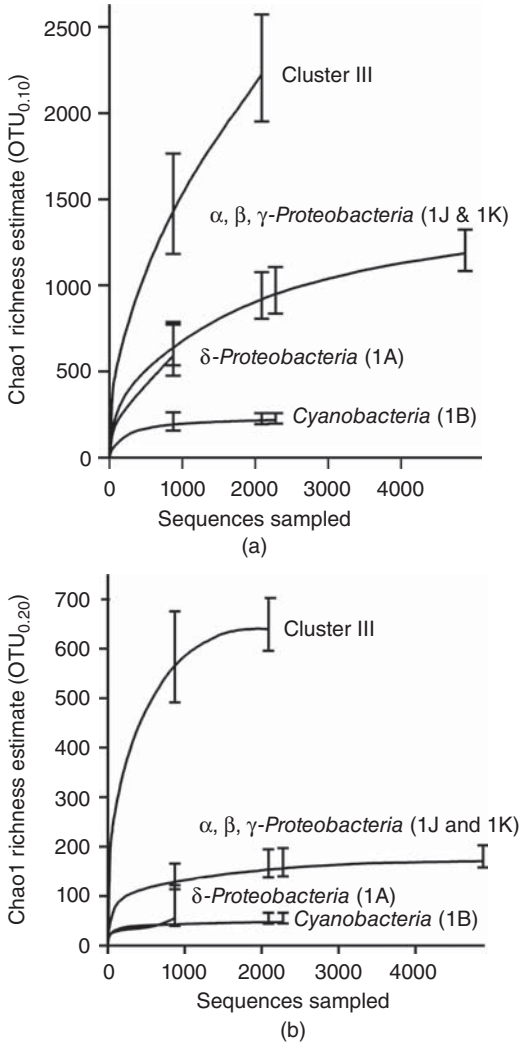

Figure 20.7 Chao1 richness estimates at a cutoff of $OTU_{0.10}$ (a) and $OTU_{0.20}$ (b) for the phylogenetic groups labeled in the figure. Bars indicate 95% confidence intervals at the sampling endpoints for each group.

employ shotgun sequencing. Diazotroph diversity can also be assessed by the enrichment and isolation of diazotrophs from environmental samples, but it is well known that cultivation imposes a strong bias on diversity estimates (Staley and Konopka, 1985). Most cultivated nitrogen fixers are heavily concentrated in the Proteobacteria (Dos Santos et al., 2012). Hence, PCR amplification of *nifH* remains an important tool for assessing diazotroph diversity.

For genes such as *nifH* that exhibit a moderate-to-high level of nucleotide variation across taxa, a degenerate primer is required to obtain "universal" coverage of taxa. The first degenerate *nifH* primer set to be designed, nifH2/nifH1 (Zehr and McReynolds, 1989), was designed primarily to target marine sequences, but since these primers target highly conserved regions of the *nifH* gene they still perform well when evaluated against a wide diversity of *nifH* sequences hitting 92% of 23847 *nifH* sequences (Gaby and Buckley, 2012). However, not all universal *nifH* primers are equally effective (Gaby and Buckley, 2012).

At least 53 universal *nifH* primers have been employed in the evaluation of *nifH* diversity (Gaby and Buckley, 2012).

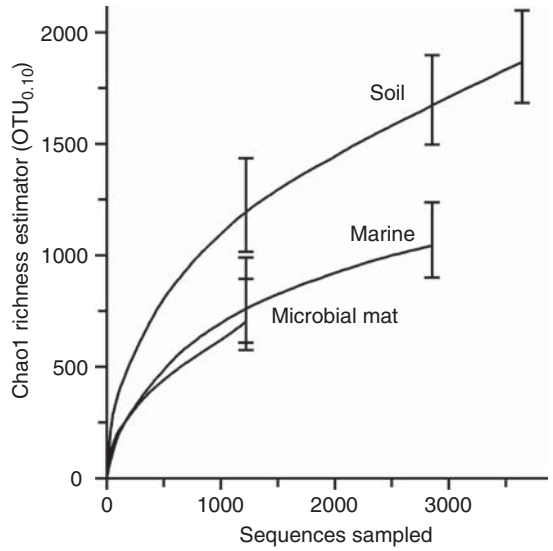

Figure 20.8 Chao1 richness estimates at an $OTU_{0.10}$ cutoff for the habitats labeled in the figure. Bars indicate 95% confidence intervals at the sampling endpoints for each group.

These primers were evaluated against the *nifH* database. While accounting for partial *nifH* sequence fragments, a total of 15 primers were found to target more than 90% of *nifH* sequences. In contrast, 23 primers were found to target less than 50% of *nifH* sequences (Gaby and Buckley, 2012). However, primers are used in pairs, and the coverage of the pair is the union of the coverage of each individual primer. Here, we report universal primer sets with high coverage as well as the commonly used primer set PolF/PolR (Table 20.2). The primer sets vary somewhat in their coverage within clusters and subclusters (Table 20.2). This result suggests that certain primer sets may be better suited to particular environments. It should be noted that this *in silico* approach to assessing primer coverage assumes that all template mismatches along the primer have equal impacts on amplification efficiency. In reality, however, mismatch position within a PCR primer can greatly affect priming efficiency (Bru et al., 2008).

While the *in silico* results are informative, the true test of primer efficacy comes at the laboratory bench, and so the primers were evaluated against a series of DNA samples from known nitrogen fixers and soils. Not all of the primer sets performed equally well in the empirical test despite having high coverage (Table 20.2). The IGK3/DVV primer set gave the best amplification from the widest diversity of templates (Gaby and Buckley, 2012). The F2/R6 primer set, despite having better *in silico* coverage than the IGK/DVV primer set, performed somewhat worse than IGK3/DVV (Table 20.2). The IGK3/DVV combination was not evaluated *in silico*, but the IGK3 primer has higher individual coverage than the IGK primer, and thus the coverage of the IGK3/DVV set is expected to be higher than IGK/DVV.

20.8 CONCLUSIONS

It is clear that the diversity of nitrogen-fixing organisms remains poorly characterized particularly with respect to

Table 20.2 Theoretical target group coverage and empirical results obtained for universal *nifH* primer sets

				Target Group Coverage*						Empirical Results†								
Primer set‡	Pos§	Len**	AT††	*nifH*	Pr	Cy	1A	III	IV	Dv	Gu	Av	Fs	Xa	Rs	Ec	AS	NT
Ueda19F/407R	19–407	389	46	86	86	100	100	82	48	+	ns	+	−	+	−	ns	s	−
IGK/DVV	31–413	383	58	83	84	85	86	68	70	+	+	+	+	+	+	−	+	−
F2/R6	115–473	359	51	95	95	98	98	84	13	−	+	+	−	−	ns	−	+	−
nifH2/nifH1	115–476	362	46	92	91	96	94	88	11	ns	+	+	−			ns	s	−
PolF/PolR	115–476	362		25	30	2	32	11	0									

*Target group coverage indicates the percentage of each target group in the *nifH* database that is hit by each universal *nifH* primer pair. Target group abbreviations are as follows: all *nifH* sequences (***nifH***), proteobacterial sequences from subclusters 1J and 1K (**Pr**), cyanobacterial sequences (**Cy**), subcluster 1A sequences (**1A**), cluster III sequences (**III**), and non-*nifH* cluster IV sequences (**IV**).

†DNA samples used for empirical tests of PCR primer pair specificity are as follows: *Desulfovibrio vulgaris* – cluster III (**Dv**), *Geobacter uraniireducens* – subcluster 1A (**Gu**), *Azotobacter vinelandii* – proteobacterial subclusters IJ and IK (**Av**), *Frankia* sp. CcI3 – subcluster 1D (**Fs**), *Xanthobacter autotrophicus* – proteobacterial subclusters IJ and IK (**Xa**), *Rhodobacter sphaeroides* – proteobacterial subclusters IJ and IK (**Rs**), *Escherichia coli* – negative control (**Ec**), agricultural soil (**AS**), and no-template control (**NT**). The symbols used to describe empirical results are product of correct size (+), no product produced (−), nonspecific amplification producing multiple bands or a single band of the wrong size (**ns**), a smeared band of indiscriminate size overlapping in size with the expected product (**s**). Note that the blank cells indicate that the evaluation was not performed.

‡Primer pairs are Ueda19F/407R (Ueda et al., 1995), IGK/DVV (Ando et al., 2006), F2/R6 (Marusina et al., 2001), nifH2/nifH1 (Zehr and Mcreynolds, 1989), and PolF/PolR (Poly et al., 2001).

§*nifH* position (**Pos**) is referenced to *Azotobacter vinelandii nifH* (GenBank ACCN# M20568).

Amplicon length in nucleotides (Len**).

††PCR annealing temperature (**AT**) in °C.

anaerobic nitrogen fixers in *nifH* cluster III. Although the continuation of molecular surveys may eventually result in a comprehensive list of extant nitrogen-fixing taxa, most *nifH* OTUs are not represented by cultivated isolates, and hence the full breadth of physiological diversity among diazotrophs remains unconstrained (Gaby and Buckley, 2011). Diazotroph ecology, while commonly studied in the photic zone of the ocean and in arable soils, remains poorly characterized in many habitats. Studies of diazotroph ecology require careful selection and application of appropriate molecular tools and the availability of well-curated reference databases (Gaby and Buckley, 2014). The emergence of low-cost sequencing technologies and further development of molecular tools for characterizing the distribution and diversity of *nifH* in the environment can be expected to vastly improve our knowledge of nitrogenase diversity in the years to come.

ACKNOWLEDGMENTS

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Section 5

Genomics of Nitrogen Fixing Organisms

Chapter 21

Genetic Regulation of Symbiosis Island Transfer in *Mesorhizobium loti*

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21.1 DISCOVERY AND FIELD TRANSFER OF THE *Mesorhizobium loti* SYMBIOSIS ISLAND

The symbiosis island of *Mesorhizobium loti* is a mobile genetic element discovered in New Zealand through its ability to convert native nonsymbiotic mesorhizobia to symbionts of the pasture legume *Lotus corniculatus* (Birdsfoot trefoil) (Sullivan et al., 1995; Sullivan and Ronson, 1998). A field trial to determine the agricultural efficacy of *L. corniculatus* in acidic tussock grassland soils was established using seed coated with the symbiont strain *M. loti* ICMP3153. The site lacked native rhizobia capable of nodulating *L. corniculatus* as *Lotus* species had not been grown there previously. Following a 7-year period, up to 19% of *L. corniculatus* nodules were found to have been formed by a diverse group of native mesorhizobial species that had acquired the capacity for symbiosis with *L. corniculatus* (Sullivan et al., 1995). Investigation at the molecular level revealed that these newly evolved *M. loti* symbionts had acquired a ~500-kb chromosomal region encoding genes involved in nodulation and nitrogen fixation (Sullivan and Ronson, 1998; Sullivan et al., 2002). This chromosomal element encoded a phage-related integrase at one end, was inserted adjacent to the *M. loti* phe-tRNA gene, and was flanked by perfect direct repeat sequences, features common to pathogenicity islands. Due to these similarities, it was named the "symbiosis island" (Sullivan and Ronson, 1998).

In subsequent field experiments in which *M. loti* strain R7A (a descendant of the ICMP3153 strain reisolated from the original field experiment) was again introduced into a site devoid of natural symbionts, it was found that over a 4-year period 75% of root nodules were formed by diverse native mesorhizobial species that had acquired the symbiosis island, although the original inoculant strain R7A was still present in the rhizosphere of the plants. Diverse symbionts were first detected 2 years after inoculation (Fig. 21.1; G. Stuart, J. Sullivan and C. Ronson, unpublished data). A similar phenomenon has been documented in Western Australian soils, where a symbiosis island identified in *M. ciceri* bv. *biserrulae* strain WSM1271 was found to transfer to indigenous mesorhizobia. The evolved symbionts were able to supplant strain WSM1271 in nodules, but in this case the evolved symbionts exhibited suboptimal nitrogen-fixing ability, negatively impacting plant growth (Beaber et al., 2002). These events suggest that there are selective advantages other than just nitrogen fixation and symbiosis that ultimately favor the evolved isolates in these soils, at least during the relatively short time-frame of these studies.

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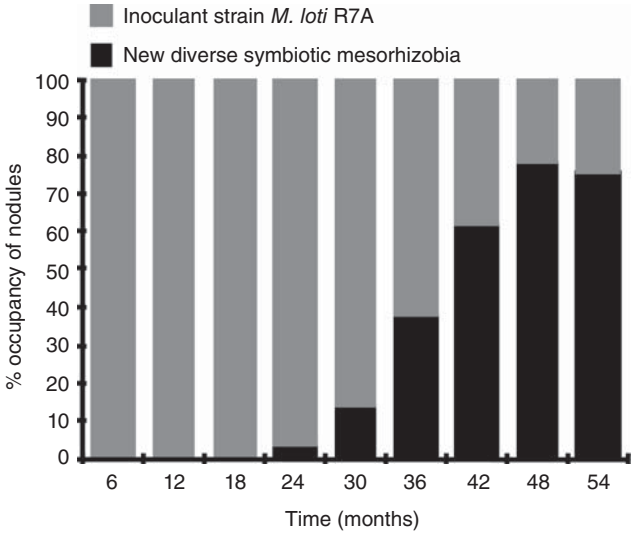

Figure 21.1 Kinetics of nodule formation by symbionts that have acquired the symbiosis island from *M. loti* strain R7A in the field.

21.2 INTEGRATION AND EXCISION OF THE SYMBIOSIS ISLAND

The symbiosis island (ICE*Ml*Sym^{R7A}) of *M. loti* strain R7A belongs to the widespread group of mobile genetic elements called integrative and conjugative elements (ICEs) (Burrus and Waldor, 2004). The integration of ICE*Ml*Sym^{R7A} DNA into the host chromosome requires an integrase IntS that catalyses recombination between 17-bp sequences that form a direct DNA repeat flanking the integrated ICE*Ml*Sym^{R7A} (Ramsay et al., 2006; Sullivan and Ronson, 1998) (Fig. 21.2). The 17-bp direct repeat sequences and surrounding regions, collectively named attachment (*att*) sites, are required for both integration and excision. The attachment sites *attL* and *attR* flank the integrated form of ICE*Ml*Sym^{R7A}, while *attP* and *attB* are used to denote the circularized ICE*Ml*Sym^{R7A} and bacterial *att* sites, respectively. The excision of ICE*Ml*Sym^{R7A} leads to the formation of *attP* and *attB*, which can be detected using polymerase chain reaction (PCR). The frequency of excision of ICE*Ml*Sym^{R7A} in the cell population can be quantified, using quantitative polymerase chain reaction (QPCR), to estimate the abundance of *attP* and *attB* relative to the total number of chromosomes present. In aerated liquid culture conditions (TY broth), the excision products *attP* and *attB* are detected in ~0.06% of cells in exponentially growing cultures and in ~6% of stationary-phase cells. Using the QPCR technique, it was discovered that efficient excision of ICE*Ml*Sym^{R7A} requires the expression of the recombination directionality factor *rdfS*, which is encoded in a gene cluster carrying genes involved in conjugation (including *rlxS*), presumably coordinating these processes. Overexpression of *rdfS* results in the loss of ICE*Ml*Sym^{R7A} from *M. loti*, producing a nonsymbiotic strain (R7ANS) (Ramsay, et al., 2006).

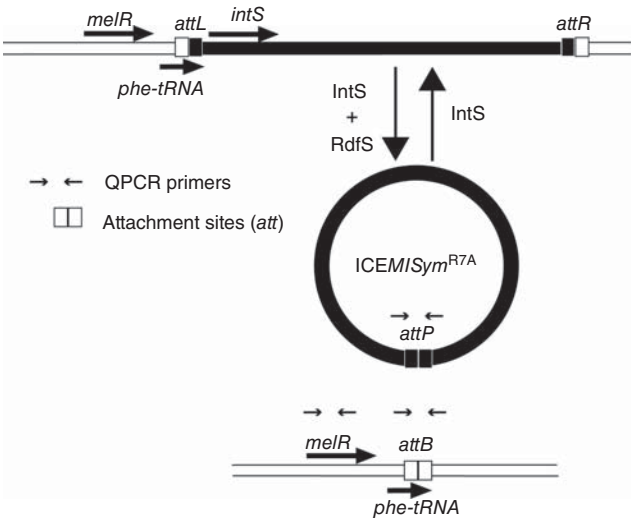

Figure 21.2 Schematic of integration and excision of ICE*Ml*Sym^{R7A}.

21.3 SYMBIOSIS ISLAND TRANSFER IS REGULATED BY QUORUM SENSING

Bacterial cell–cell communication or "quorum sensing" (QS) regulates numerous phenotypes in rhizobia, including horizontal gene transfer. QS-induced plasmid transfer has been characterized in both Gram-negative and Gram-positive bacterial species. For some plasmids, the QS regulatory factors and their cognate signaling molecules (sometimes referred to as pheromones or quoromones) can specifically induce transfer between plasmid-carrying and plasmid-free cells (Danino et al., 2003; Dunny, 2007; Wisniewski-Dye and Downie, 2002; see Chapter 37). ICE*Ml*Sym^{R7A} encodes a QS system related to that of the Ti plasmid of *Agrobacterium tumefaciens* and pRL1JI of *Rhizobium leguminosarum* (Ramsay et al., 2009; Ramsay, et al., 2006). In the plasmid systems, quorum-dependent transcriptional activation is carried out by a DNA-binding protein TraR. In the presence of the membrane-diffusible autoinducer molecule *N*-(3-oxooctanoyl)-L-homoserine lactone (3-oxo-C8-HSL), TraR is able to activate transcription of transfer genes and transcription of the 3-oxo-C8-HSL synthase-encoding gene *traI* in a positive-feedback loop (Frederix and Downie, 2011).

The TraR protein of ICE*Ml*Sym^{R7A} shares 29% and 32% amino acid identity with the Ti and pRL1JI plasmids, respectively, while the plasmid TraR proteins share 29% identity, suggesting that the three systems share a similar level

of evolutionary divergence. ICE*Ml*Sym^{R7A} also encodes two predicted *N*-acyl homoserine lactone (AHL) synthase genes *traI1* and *traI2*. TraI1 predominantly produces *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL), the cognate signal of ICE*Ml*Sym^{R7A} TraR. The predicted TraI2 protein contains all conserved residues known to be involved in catalysis for the LuxI/TraI AHL synthase family; however, no functional role or autoinducer synthase activity has been detected (Ramsay et al., 2009). The TraI1 and TraI2 sequences are more related to each other than they are to other database sequences, suggesting that *traI2* may be the product of a gene duplication event. It is possible that *traI2* has become a pseudogene, or it may be involved in the production in of an as-yet unknown signaling molecule. The TraR protein of ICE*Ml*Sym^{R7A} activates transcription of the *traI1* and *traI2* genes on ICE*Ml*Sym^{R7A}, resulting in a positive-feedback loop through activation of TraI1 expression and 3-oxo-C6-HSL production (Fig. 21.3). Predicted TraR-binding sites (*tra*-boxes) are located upstream of both *traI1* and *traI2* (Ramsay, et al., 2009).

Introduction of a plasmid-borne copy of ICE*Ml*Sym^{R7A} *traR* (pJRTraR) results in the excision and stable maintenance of ICE*Ml*Sym^{R7A} in 100% of cells, a 1000-fold increase in the production of 3-oxo-C6-HSL and a 40-fold increase in conjugative transfer (Ramsay et al., 2013; Ramsay et al., 2009; Ramsay et al., 2006). In contrast, addition of exogenous AHLs to high levels has no effect on these properties, suggesting that expression of *traR* or TraR activity is usually suppressed. Interestingly, the stable maintenance of ICE*Ml*Sym^{R7A} in the presence of pJRTraR requires a functional copy of *rlxS*, which encodes the ICE*Ml*Sym^{R7A} relaxase protein required for conjugation. This suggests that while ICE*Ml*Sym^{R7A} is excised, it replicates via rolling-circle replication, a feature that has been subsequently observed for ICEs in Gram-positive species (Thomas et al., 2013). The notion that an ICE is able to be stably maintained via an extrachromosomal replication mechanism challenges the assumption that excision is merely a transient event (Burrus et al., 2002). Indeed, recent studies of ICEs in *Pseudomonas* suggest that a minority of ICE-carrying cells may differentiate (sometimes terminally) into nongrowing "mating bodies" in which ICEs remain excised and conjugate to other cells (Reinhard et al., 2013).

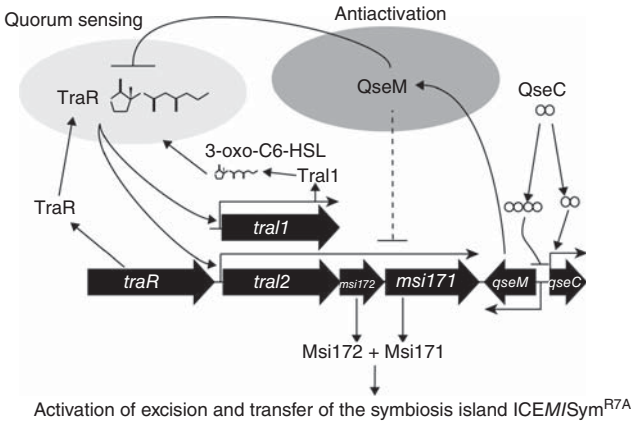

Figure 21.3 Regulation of ICE*Ml*Sym^{R7A} by quorum sensing and antiactivation.

21.4 QUORUM SENSING INDUCES A NOVEL ACTIVATOR OF SYMBIOSIS ISLAND EXCISION AND TRANSFER

The increased 3-oxo-C6-HSL production observed in cells carrying pJRTraR results from transcriptional activation of *traI1* and autoinduction by TraR-3-oxo-C6-HSL. The activation of excision and conjugative transfer by TraR requires transcriptional activation of the *traI2-msi172-msi171* operon and, specifically, the presence of *msi172* and *msi171*. Nonpolar deletions in *msi172* or *msi171* result in a total loss of excision and conjugative transfer but do not affect AHL production (Ramsay et al., 2009), effectively uncoupling QS from the regulatory cascade. The predicted protein products of *msi172* and *msi171* belong to the large uncharacterized protein family DUF2285, conserved on ICE*Ml*Sym^{R7A}-related elements found throughout the Proteobacteria. Our molecular investigations suggest that *msi172* and *msi171* work together to directly activate expression of *rdfS* promoter, indicating they may encode factors involved in transcriptional activation (Ramsay et al., 2013; Ramsay et al., 2009). Comparisons between related ICE elements reveal that the QS genes *traR* and *traI1/2* are infrequently associated with *msi172-msi171* and *rdfS*, suggesting that addition of QS regulation to this circuit is a recent evolutionary event (Ramsay, et al., 2013).

21.5 THE QS CIRCUIT IS CONTROLLED BY AN ANTIACTIVATOR

The large increase in AHL production and ICE*Ml*Sym^{R7A} excision and transfer that results from ectopic *traR* expression through pJRtraR indicates that the stoichiometry of TraR with other negative regulatory factor(s) is critical for the observed suppression of the QS autoinduction circuit in wild-type cells. On the Ti and pRL1JI plasmids, the TraM protein inhibits QS through direct antiactivation of the TraR protein (Fuqua et al., 1995). *M. loti* lacks TraM; however, ICE*Ml*Sym^{R7A} encodes an unrelated antiactivator of TraR, the QseM protein. The *qseM* gene is encoded downstream of and transcribed toward *traR* in a configuration similar to that of the *traM* gene on pRL1JI. Deletion of *qseM* results in activation of 3-oxo-C6-HSL production, and ICE*Ml*Sym^{R7A}

excision and conjugation, essentially producing a phenocopy of a strain carrying pJRTraR (Ramsay et al., 2013). Mutation of *traR* in the *qseM*-deleted strain results in a loss of this activation, confirming the dependence of this activation on TraR. Bacterial two-hybrid analysis has demonstrated that QseM can directly interact with the TraR protein in a heterologous *Escherichia coli* background. However, the interaction with TraR appears to be AHL-dependent, unlike that observed between TraR and TraM, also in *E. coli*. This suggests that the mechanism of TraR antiactivation by QseM may be distinct. QseM shows no sequence similarity to TraM; however, it is similar in size and our preliminary analysis of purified QseM suggests that like TraM it is α-helical in structure (Ramsay et al., 2013; J. Ramsay, A. Major, K. Krause and C. Ronson, unpublished data).

Overexpression of QseM suppresses excision of ICE*Ml*Sym^{R7A} excision (<0.1%) to levels significantly below that observed in wild-type cultures. Surprisingly however mutation of *traR* has only a marginal effect on the frequency of ICE*Ml*Sym^{R7A} excision, although it does result in significantly lowered rates of conjugative transfer (Ramsay et al., 2013; Ramsay et al., 2009). This is a clear evidence that QseM has additional targets other than TraR. Interestingly, the QseM protein contains weak but convincing sequence similarity to the DUF2285 family (and similarity to the protein product of *msi171*), and several QseM homologs are annotated as belonging to this family. It is possible that an ancestor of the QseM family of proteins diverged from the DUF2285 family and evolved antagonistic activity against excision-activating relatives such as Msi172 and Msi171 (Ramsay et al., 2013). We are currently investigating the possibility that Msi172 and Msi171 are the secondary targets of QseM.

21.6 A MOLECULAR SWITCH CONTROLS THE PRODUCTION OF THE ANTIACTIVATOR

Our dissection of the regulatory circuits controlling ICE*Ml*Sym^{R7A} excision and transfer has revealed that there is a delicate balance between repression by the QseM protein and runaway positive autoinduction of the QS system. This implies that at the top of the regulatory hierarchy, the switch to the excised and transferring state is controlled by the stoichiometry of TraR-3-oxo-C6-HSL and QseM. Our analyses of *traR* expression indicate that it is transcribed at a low basal level throughout growth, and that both *traR* transcriptional activation and mRNA abundance are unaffected by activation of QS or the presence of QseM (Ramsay et al., 2013). It remains possible that the expression of *traR* is activated by some as yet undiscovered factor. More however is understood about the expression of *qseM*, which is controlled by the DNA-binding protein QseC.

The *qseC* gene is divergently transcribed from *qseM* as a leaderless mRNA. QseC binds two adjacent operator sequences between *qseM* and *qseC* and is able to both activate its own expression and repress expression of *qseM* (Fig. 21.4). QseC binds the *qseM*-proximal operator O_L at low concentrations, while the *qseC*-proximal operator O_R is only bound in the presence of QseC-bound O_L. This suggests that with increasing QseC concentration, O_L is initially bound and that O_R is bound secondarily in a cooperative manner. The -35 region of the *qseC* promoter is located immediately downstream of the O_L operator sequence, consistent with positive autoinduction of *qseC* expression through recruitment of the RNA polymerase holoenzyme (RNAP) to the -35 site within O_R. The *qseM*-35 region is

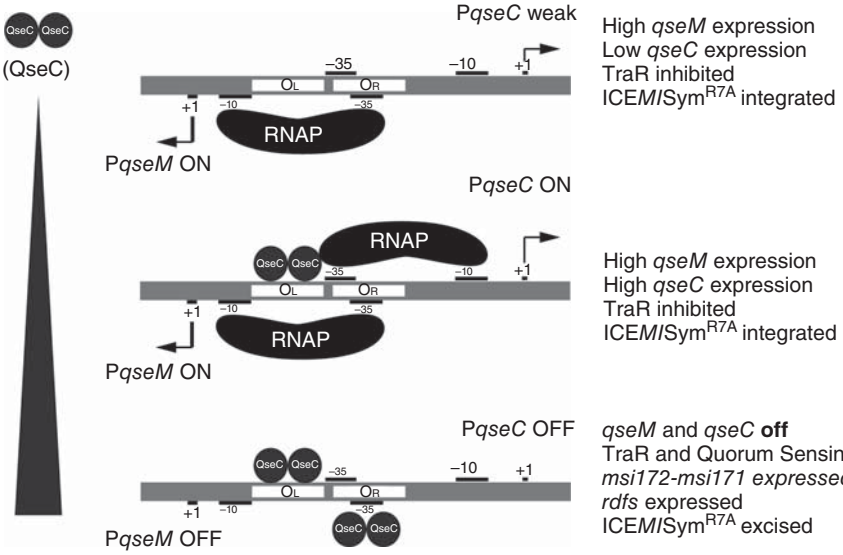

Figure 21.4 Regulation of *qseC* and *qseM* expressions by the QseC switch.

also within O_R, however, due to the divergent orientation of *qseM*, the access of RNAP to the *qseM* promoter is likely blocked by QseC binding to O_R, consistent with the observed increase in *qseM* expression in a *qseC* mutant. In a *qseC* mutant, pJRTraR is unable to induce 3-oxo-C6-HSL production or elevate the frequency of excision and conjugative transfer. Consistent with our model, this suggests that in the absence of QseC, QseM dominates the balance between QseM and TraR-3-oxo-C6-HSL, preventing positive autoinduction of QS. When this model is considered with respect to the excision frequencies observed in wild-type cells (~0.06% in log phase and ~6% in stationary phase), it is tempting to conclude that in the ~6% of cells containing ICE*Ml*Sym^{R7A}, the QseC concentration is high enough to repress expression of *qseM*, resulting in the de-repression of the QS-positive induction circuit and subsequent activation of *msi172-msi171* and *rdfS* expression (Ramsay et al., 2013).

21.7 CONCLUDING REMARKS

Although ICE*Ml*Sym^{R7A} excision and transfer is regulated by QS and can occur at very high levels if the QS system is de-repressed, the further tier of regulation provided by QseM–QseC ensures that only low levels of AHL are produced by the population, and that most cells remain blind to this signal, whatever its level. As mentioned earlier, while TraR is largely dispensable for the basal frequency of excision observed in R7A, it is absolutely required for transfer. This demonstrates that TraR-activated expression and induction of transfer is occurring in R7A, albeit at a low frequency, with low population-level AHL production (Ramsay et al., 2009). Therefore, it seems plausible that the QseM–QseC switch facilitates bimodal induction of QS in the population, allowing the coordination of transfer from only the subpopulation of *qseM*-repressed cells. Other physiological factors, such as stress or the availability of suitable recipients, could introduce conditionality on the QS system and/or repression by QseM, by modulating the proportion of cells receptive to be induced by QS. This mechanism could allow ICE*Ml*Sym^{R7A} to maximize its potential for propagation from both vertical and horizontal descents by appropriately partitioning a subpopulation of cells for transfer in response to environmental conditions.

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Chapter 22

The *Azotobacter vinelandii* Genome: An Update

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22.1 INTRODUCTION

This chapter presents an updated and expanded description of the *Azotobacter vinelandii* strain DJ genome. The first description of this genome was published in 2009 (Setubal et al., 2009). In that publication, the focus was on the following sets of genes: respiration and respiratory protection genes, nitrogen-fixation-related genes, genes related to other oxygen-sensitive processes (CODH, FDH, and hydrogenases), alginate genes, and polymer production and encystment genes. Here, we update information related to genome annotation and phylogeny, and include new material that was not part of that paper because of space limitations.

We do not repeat information from the above paper related to the gene categories listed. The reader interested in learning about these genes should consult the original paper and a follow-up review that contains more information on the genomics of nitrogen fixation, with a focus on *A. vinelandii* (O'Carroll and Dos Santos, 2011).

22.2 MATERIALS AND METHODS

The phylogenetic trees were built using 20 genomes (Table 22.1). For both trees, we used OrthoMCL (Li et al., 2003) to obtain families of orthologous proteins. For the phylogeny of Pseudomonadaceae (Fig. 22.1), OrthoMCL provided 1303 families containing exactly one representative member of each ingroup genome and at most one from the outgroup genome (out of 10,026 families). Each one of these groups was aligned with Muscle (Edgar, 2004), and the noninformative columns were removed by Gblocks (Castresana, 2000). All alignments were concatenated, totaling 399,671 columns, and this final concatenated alignment was used as input to RAxML (Stamatakis, 2006) with the PROTGAMMAWAGF model to build the tree. Bootstrap support values were obtained with 100 replicates. The same method was used to build the tree in Figure 22.2, but in this case we used 6 genomes, 15 families (out of 4989), and 4421 columns. Note that only 80 *Azotobacter chroococcum* protein sequences are available in GenBank.

Gene-sharing information shown in Table 22.3 was obtained from orthoMCL results. AlienHunter (AH) (Vernikos and Parkhill, 2006) was used to identify anomalous regions in the *A. vinelandii* DJ genome. These regions have unusual sequence composition and are considered anomalous if the AH score is above that of an automatically calculated threshold, based on the background sequence composition of the whole genome. For the *A. vinelandii* DJ genome, this threshold was 13.02, and 54 anomalous regions were found. Alignment between chromosomes was carried out using the script Promer from the MUMmer package

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Table 22.1 GenBank information on genomes used to create phylogenies

Taxon Name	Taxon ID	Accession Number(s)
Azotobacter vinelandii DJ	322710	NC_012560
Azotobacter chroococcum	355	HD017153.1, HB959743.1, GN125302.1, GM953710.1
Pseudomonas stutzeri A1501	379731	NC_009434
Pseudomonas stutzeri ATCC 17588	96563	NC_015740
Pseudomonas stutzeri CCUG 29243	1196835	NC_018028
Pseudomonas stutzeri DSM 10701	1123519	NC_018177
Pseudomonas stutzeri DSM 4166	996285	NC_017532
Pseudomonas stutzeri RCH2	644801	NC_019936, NC_019937, NC_019938, NC_019939
Pseudomonas putida ND6	231023	NC_005244, NC_017986, NC_018746
Pseudomonas aeruginosa PAO1	208964	NC_002516
Pseudomonas brassicacearum NFM421	994484	NC_015379
Pseudomonas entomophila L48	384676	NC_008027
Pseudomonas fluorescens Pf-5	220664	NC_004129
Pseudomonas fulva 12-X	743720	NC_015556
Pseudomonas mendocina ymp	399739	NC_009439
Pseudomonas putida KT2440	160488	NC_002947
Pseudomonas syringae tomato DC3000	223283	NC_004578, NC_004632, NC_004633
Cellvibrio japonicus Ueda107	498211	NC_010995
Cellvibrio sp. BR	1134474	NZ_AICM00000000
Chromohalobacter salexigens DSM 3043	290398	NC_007963

(Kurtz et al., 2004). Promer performs alignments between translated nucleotide sequences. Promer was not sensitive enough to pick up the alignment between the *iscAnif* gene present in *Teredinibacter turnerae* T7901 genome and that of *A. vinelandii* DJ; this detection required a BLAST search. Clustered regularly interspaced short palindromic repeats (CRISPRs) were found using the CRISPRfinder server (Grissa et al., 2007).

22.3 RESULTS AND DISCUSSION

22.3.1 Genome Features and Phylogeny

The basic genome features of *A. vinelandii* DJ are shown in Table 22.2. They are the same as those reported previously (Setubal et al., 2009), with additional information on group II introns and CRISPRs.

>Setubal et al. (2009) presented a maximum-likelihood phylogeny of Pseudomonadaceae. Figure 22.1 presents a new phylogeny of Pseudomonadaceae, obtained by the same method. This phylogeny has the following novel features compared with the one published previously: it includes two *Cellvibrio* genomes, which are also Pseudomonadaceae but for which in 2009 there were no complete genomes available; five new *Pseudomonas stutzeri* genomes, in addition to *P. stutzeri* A1501; and some newly sequenced *Pseudomonas* species. With the exception of *P. stutzeri*, for all other *Pseudomonas* species we used just one representative per species. This phylogeny is in complete agreement with that from Setubal et al. (2009). The addition of new genomes shows that the *Pseudomonas* species separate into two basic groups: one containing all *P. stutzeri* species and the other containing the remaining *Pseudomonas* species. *A. vinelandii* DJ localizes externally to the *Pseudomonas* species but internally with respect to the two *Cellvibrio* species.

P. stutzeri A1501 is a nitrogen fixer (Yan et al., 2008). Among the five new *P. stutzeri* strains that we included in the phylogenetic inference only one other strain also fixes nitrogen: *P. stutzeri* DSM 4166, an isolate from the rhizosphere of a *Sorghum nutans* cultivar (Yu et al., 2011; see also Chapter 10).

We further investigated the phylogeny of *A. vinelandii* by using the relatively few protein sequences publicly available from *A. chroococcum*. *A. chroococcum* was the first *Azotobacter* species to be described and is commonly found in soils worldwide; it is also a nitrogen fixer (Page, 1987). The resulting phylogeny is shown in Figure 22.2. As expected, *A. chroococcum* groups with *A. vinelandii* DJ, with both species appearing separate from the *Pseudomonas* group.

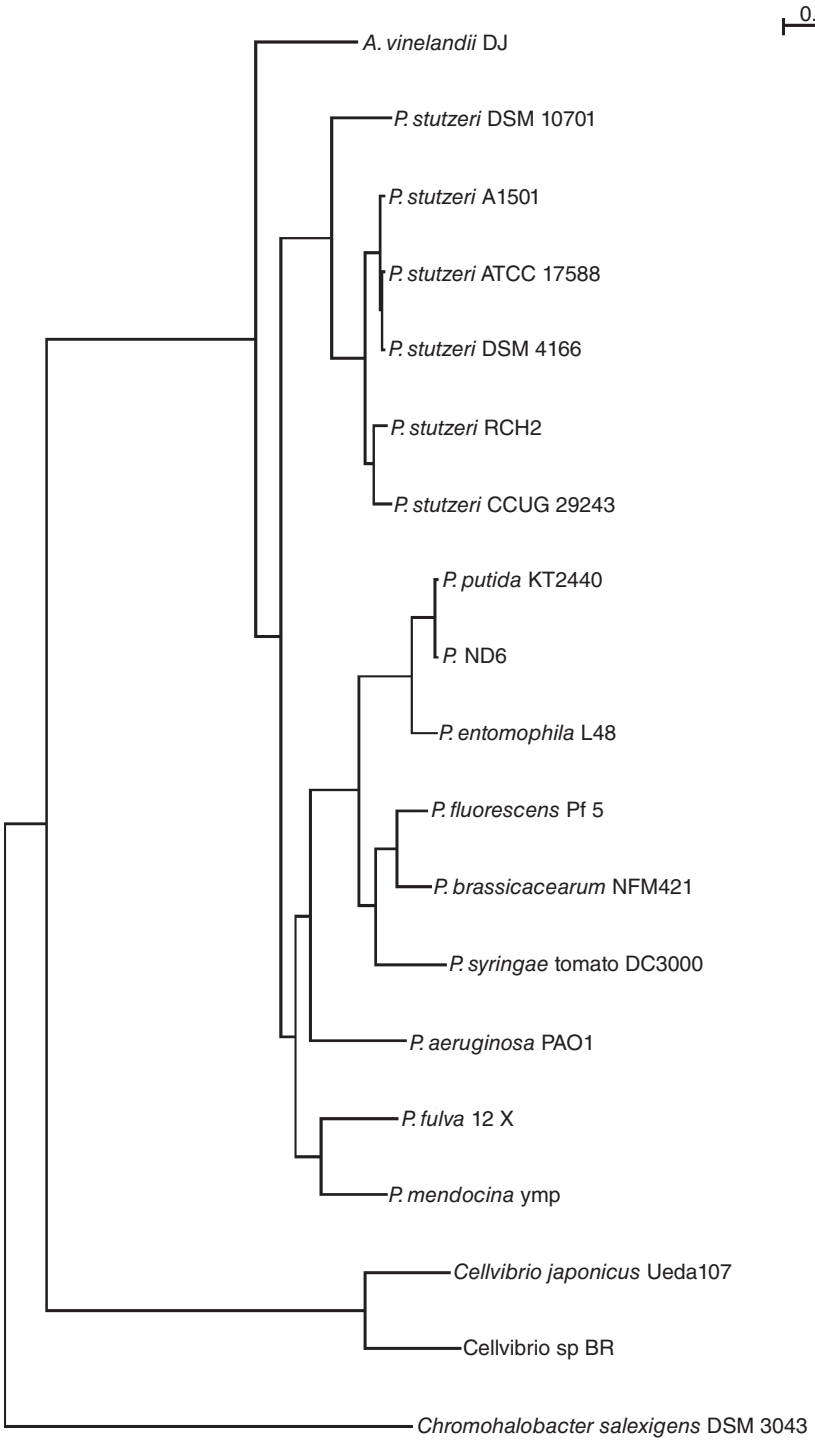

Figure 22.1 A maximum-likelihood phylogenetic tree including representatives from all Pseudomonadaceae species whose genome has been completely sequenced. *Chromohalobacter salexigens* DSM 3043 was used as outgroup. All branches received 100% bootstrap support. The scale shows the number of substitutions per site in branch lengths.

The proximity between *Azotobacter* and *Pseudomonas* shown in these trees justifies the question of whether the genus *Azotobacter* should be reclassified as *Pseudomonas*. Ozen and Ussery (2012) have studied this question, performing extensive comparative analyses. They reached the conclusion that there is "a high similarity between *A. vinelandii* and the *Pseudomonas* genus, suggesting that *Azotobacter* might actually be a *Pseudomonas*." The two trees that we present here show clearly that the two *Azotobacter* species, while related to the sequenced *Pseudomonas*, form a distinct group. Whether or not the *Azotobacter* genus should be reclassified as *Pseudomonas* is a question that we do not address here further.

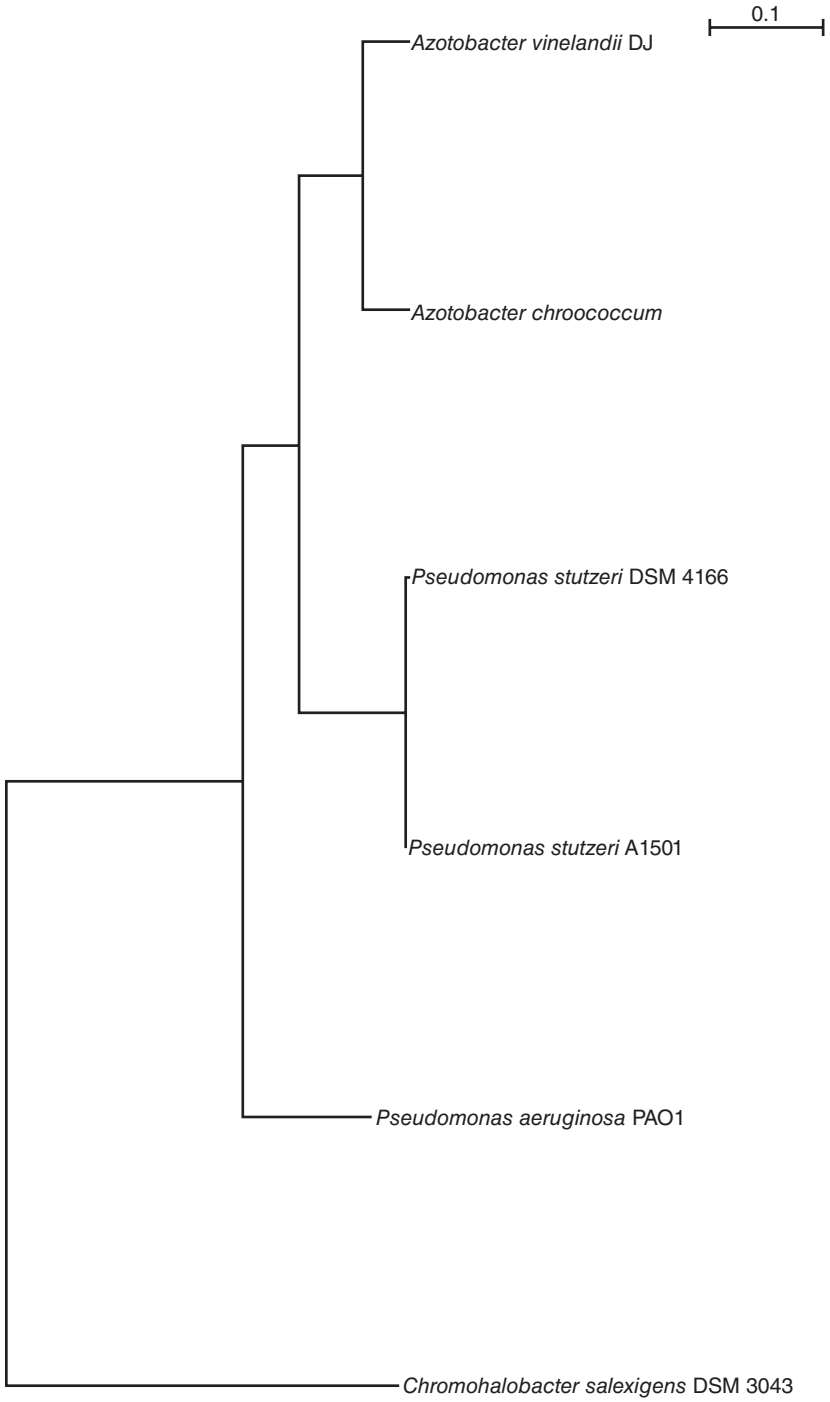

Figure 22.2 A maximum-likelihood phylogenetic tree for some Pseudomonadaceae species including *Azotobacter chroococcum*. All branches received 100% bootstrap support. The scale shows the number of substitutions per site in branch lengths.

22.3.2 Sharing of Nitrogen Fixation-Related Genes

Of all the genomes used to build the phylogeny in Figure 22.1, only *A. vinelandii* DJ, *P. stutzeri* A1501, and *P. stutzeri* DSM 4166 are diazotrophs. We have determined which genes are shared by these three genomes such that none of these genes is also present in the remaining genomes. The results are shown in Table 22.3. With the exception of transposase genes, these three genomes share exactly two regions. One is the major *nif* region (Avin_13060 to Avin_01710); the other is a region (Avin_50900 to Avin_51060) composed of two subregions: the *rnf1* region (Avin_50900 to Avin_50980) and the minor *nif* region

Table 22.2 Basic genome features of *Azotobacter vinelandi* DJ

Feature	*Azotobacter vinelandi* DJ
Size (bp)	5,365,318
%GC	65.7
Total protein-coding genes	5,051
With functional assignment	3,561 (70.5%)
Conserved hypothetical	739 (14.6%)
Hypothetical	751 (14.9%)
Pseudogenes	66
rRNA operons	6
tRNAs	64
Other RNAs	18
Group II introns	8
CRISPRs	3

(Avin_50990 to Avin_51060). Both sets of genes are related to nitrogen fixation (Curatti et al., 2005; O'Carroll and Dos Santos, 2011).

22.3.3 The Origin of Nitrogen Fixation Genes in *A. vinelandii*

There is considerable interest in the origins of nitrogen fixation (Raymond et al., 2004; Kechris et al., 2006; see also Chapters 8, 16, 20). One hypothesis is that nitrogen fixation appeared after the emergence of bacteriochlorophyll biosynthesis and then spread by horizontal gene transfer (HGT) to various microbial lineages (Boyd et al., 2011). The availability of the *A. vinelandii* DJ genome sequence and that of other related organisms presents an opportunity to investigate the issue of HGT for this particular group.

Figure 22.3 shows a graph of anomalous regions in terms of nucleotide composition of the *A. vinelandii* DJ genome. Many of these regions are candidates for having been horizontally transferred; however, none of the nitrogen-fixation genes is contained in any of these regions. This evidence, coupled with the results on gene sharing with the diazotrophic *P. stutzeri* species presented in the previous section, suggests that nitrogen-fixation genes were present in the common ancestor of *A. vinelandii* and *P. stutzeri*, having been lost in the nondiazotrophic *P. stutzeri* species. This means, in turn, that if HGT indeed took place in this lineage, it happened either in that ancestor or earlier.

In order to explore this question further, we have determined which organisms share nitrogen-fixation genes with high similarity to those of *A. vinelandii* and *P. stutzeri*. We carried out this by running BLAST (Altschul et al., 1997) searches using as queries the protein sequences for *A. vinelandii* DJ genes *nifHDKENB* against the nr database from GenBank. These genes have been suggested as a minimum gene set for the computational identification of diazotrophs from whole genome sequences (Dos Santos et al., 2012). Partial results can be seen in Table 22.4. *T. turnerae* T7901 is shown because it gave the best (by bitscore) BLAST hit for all six query sequences among the non-Pseudomonadaceae.

T. turnerae is a marine intracellular endosymbiont Gammaproteobacterium in the order Alteromonadales (NCBI taxonomy). The genome of strain T7901 has been sequenced (Yang et al., 2009), and the similarity of its nitrogen-fixation genes to those of *Azotobacter* and *P. stutzeri* was noted in that publication. Yang et al. (2009) suggested that "the *nif* cluster in *T. turnerae* was acquired via horizontal gene transfer from a *Pseudomonas*-like bacterium." In this context we note that, similar to the *A. vinelandii* results above, none of the *nif* genes in *T. turnerae* T7901 lie in an anomalous region (data not shown). Moreover, an alignment of the *A. vinelandii* DJ genome with that from *T. turnerae* T7901 shows that there is local synteny between the major *nif* regions in both genomes and between the minor *nif* regions in both genomes (Figs. 22.4 and 22.5). In the case of the major *nif* region, the *T. turnerae* chromosome contains an insertion and a deletion with respect to the *A. vinelandii* sequence (Fig. 22.4). The insertion runs from position 1,603,694 to position 1,625,049 in *T. turnerae* genome coordinates and contains genes unrelated to nitrogen fixation, some of which are found scattered in the *A. vinelandii* DJ genome. The deletion occurs between *A. vinelandii* genome coordinates 148,689 and 153,036 (or from Avin_01540 to Avin_01600). This region includes five hypothetical proteins and one ATP-binding cassette (ABC) transporter. This deletion suggests that these genes may not be strictly necessary for nitrogen fixation. In the case of the second region (Fig. 22.5), it essentially corresponds to the second shared region between *A. vinelandii* DJ and the two *P. stutzeri* diazotroph genomes already noted earlier, which includes the minor *nif* region.

These alignments (as well as the global alignment between the two chromosomes – data not shown) are characteristic of genomes that share a common ancestor but which have diverged for a long enough time such that many rearrangements have taken place, but still preserving small syntenic blocks. These results suggest that it is more likely that *T. turnerae*, *A. vinelandii*, and *P. stutzeri* inherited their nitrogen-fixation genes from a common ancestor. If this hypothesis is correct, then we can ask the question: which ancestor, if any, acquired its nitrogen-fixation genes through HGT? Our BLAST search did reveal a few more interesting links, as described next.

We investigated 15 additional non-Pseudomonadaceae species in the BLAST search, selected using the following criteria: only species containing at least five of the six minimum-set genes were selected; and all alignments had to have at least 60% amino acid identity (Table 22.5). Of

Table 22.3 Genes shared between the *A. vinelandii* DJ, *P. stutzeri* A1501, and *P. stutzeri* DSM 4166 genomes and absent in the other fully sequenced Pseudomonadaceae

Gene (locus tag)	Gene name	Product
Avin_01360	—	Hypothetical protein
Avin_01370	—	Hypothetical protein
Avin_01380	*nifH*	Nitrogenase iron protein
Avin_01390	*nifD*	Nitrogenase molybdenum-iron protein alpha chain: nitrogenase component I, alpha chain
Avin_01400	*nifK*	Nitrogenase molybdenum-iron protein subunit beta
Avin_01410	*nifT*	Nitrogen-fixation protein
Avin_01420	*nifY*	Nitrogenase iron-molybdenum cofactor biosynthesis protein
Avin_01430	—	Hypothetical protein
Avin_01440	*lrv*	Nitrogen-fixing leucine-rich variant repeat 4Fe–4S cluster protein
Avin_01450	*nifE*	Nitrogenase MoFe-cofactor biosynthesis protein
Avin_01470	*nifN*	Nitrogenase molybdenum-cofactor biosynthesis protein
Avin_01480	*nifX*	Nitrogenase MoFe-cofactor biosynthesis protein
Avin_01490	—	Hypothetical protein
Avin_01500	—	Hypothetical protein
Avin_01510	—	Nitrogen fixation (4Fe–4S) ferredoxin-like protein
Avin_01520	*feS1*	Nitrogen fixation (2Fe–2S) ferredoxin (Shethna I protein)
Avin_01530	—	Hypothetical protein
Avin_01540	—	Hypothetical protein
Avin_01550	—	Hypothetical protein
Avin_01560	—	Hypothetical protein
Avin_01570	—	Hypothetical protein
Avin_01610	*iscAnif*	Nitrogen fixation Fe–S cluster assembly protein
Avin_01620	*nifU*	Nitrogen fixation Fe–S cluster scaffold protein
Avin_01630	*nifS*	Nitrogen fixation cysteine desulfurase
Avin_01650	*cysE1*	Nitrogen fixation serine O-acetyltransferase
Avin_01660	—	Hypothetical protein
Avin_01670	*nifW*	Nitrogen fixation protein
Avin_01680	*nifZ*	Nitrogen fixation protein
Avin_01690	*nifM*	Nitrogen fixation cis-trans peptidyl prolyl isomerase
Avin_01700	*clpX*	ATP-dependent protease ATP-binding subunit
Avin_01710	*nifF*	Flavodoxin
Avin_09770	—	Transposase IS3/IS911 (5 copies in Avin)
Avin_50900	—	Nitrogen fixation-like protein
Avin_50910	*nafY*	Nitrogen fixation-like protein subunit gamma
Avin_50920	*rnfH*	RnfABCDGE type electron transport complex subunit H
Avin_50930	*rnfE1*	RnfABCDGE type electron transport complex subunit E
Avin_50940	*rnfG1*	RnfABCDGE type electron transport complex subunit G
Avin_50950	*rnfD1*	RnfABCDGE type electron transport complex subunit D
Avin_50960	*rnfC1*	RnfABCDGE type electron transport complex subunit C
Avin_50970	*rnfB1*	RnfABCDGE type electron transport complex subunit B
Avin_50980	*rnfA1*	RnfABCDGE type electron transport complex subunit A
Avin_50990	*nifL*	Nitrogen fixation regulatory protein
Avin_51010	*nifB*	Nitrogenase cofactor biosynthesis protein
Avin_51020	—	Ferredoxin protein
Avin_51030	—	Nitrogenase-associated protein
Avin_51040	*nifO*	Nitrogen fixation cofactor assembly protein
Avin_51050	*nifQ*	Rhodanese/sulfurtransferase-like protein
Avin_51060	—	Glutaredoxin-like protein

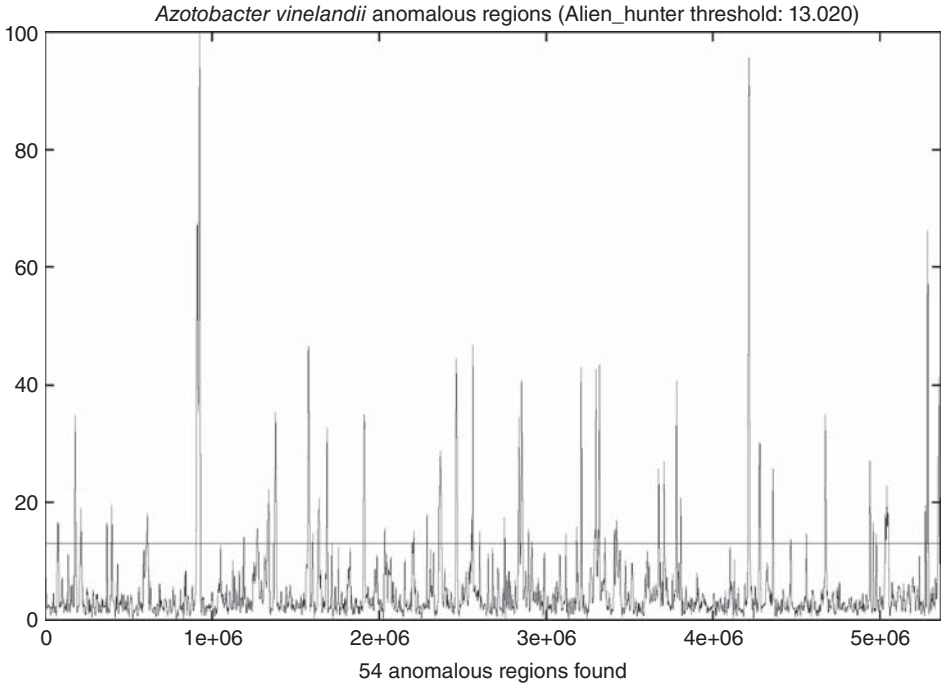

Figure 22.3 Anomalous regions in the genome of *A. vinelandii* DJ genome. The *x*-axis represents genome coordinates and the *y*-axis represents AlienHunter scores, calculated for each 5 kbp-length window with an offset of 2.5 kbp.

these 16 species (including *T. turnerae*), 12 are Gammaproteobacteria (with seven species in the order Chromatiales), and four are Betaproteobacteria.

Our interpretation of these results is that the presence of nitrogen-fixation genes in *A. vinelandii* is the result of vertical inheritance up to an unidentified Gammaproteobacterium ancestor. Because that hypothesized ancestor is also the ancestor of many nondiazotrophs, such a hypothesis requires that nitrogen-fixation genes were lost in many descendant lineages of that ancestor. The above-mentioned evidence suggests that HGT may have happened between that Gammaproteobacterium ancestor and a Betaproteobacterium ancestor, given that the connection between these two groups was one of the "highways" for HGT proposed by Beiko et al. (2005). Additional research is required to determine whether nitrogen-fixation genes were indeed exchanged in ancient HGT events between Betaproteobacteria and Gammaproteobacteria.

Table 22.4 Similarity of *A. vinelandii* DJ protein sequences given by percent identity with sequences from the following organisms: *P. stutzeri* A1501, *P. stutzeri* DSM 4166, and *Teredinibacter turnerae* T7901

nif Gene	A1501	DSM 4166	*Teredinibacter turnerae* T7901
H	92	92*	90
D	91	91	86
K	91	90	82
E	89	88	83
N	81	80	67
B	86*	86	89

The results were obtained with BLAST. For those marked with an asterisk, it was necessary to use tBLASTn to obtain the correct result because of annotation problems. All alignments covered 100% or nearly 100% of both query and subject sequences.

22.3.4 Other Features of the *A. vinelandii* DJ Genome

The *A. vinelandii* DJ genome has the remarkable property that it contains eight group II introns (Fig. 22.6), more than any other bacterial genome (Dai et al., 2003). (The database on which this statement is based was last updated in 2008.) Group II introns are a class of RNAs that can perform a self-splicing reaction. Bacterial group II introns almost always encode reverse transcriptase ORFs (Open Reading Frames) and are active mobile elements (Dai and Zimmerly, 2002). All of the identified group II introns in *A. vinelandii* DJ encode reverse transcriptase ORFs. These introns have been carefully annotated and are part of the *A. vinelandii* DJ GenBank record.

Another notable feature of the *A. vinelandii* DJ genome is that it contains three sets of CRISPRs. CRISPRs are thought to constitute a kind of RNA-interference-based immune system for prokaryotes (Makarova et al., 2006). We report them here primarily because they are not currently annotated in the *A. vinelandii* DJ GenBank record. Two of the sets display the expected structure for CRISPRs: from position 3261022 to position 3263057, with 30 spacers, and from position 3265050 to position 3266352, with 19 spacers. A third set has "questionable structure" (Grissa et al., 2007),

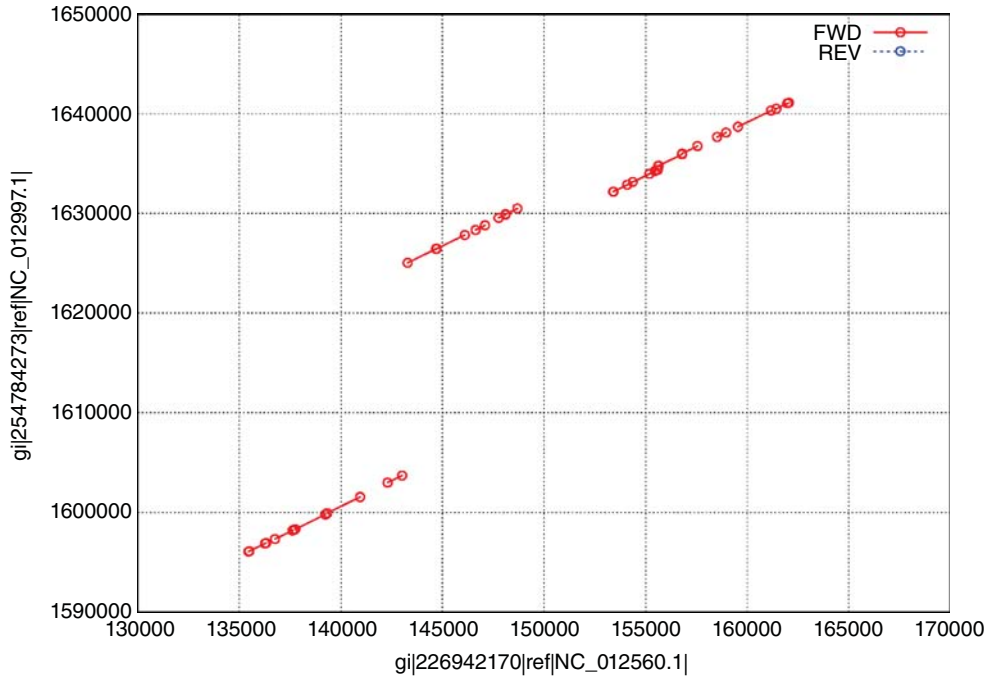

Figure 22.4 Alignment between the chromosome sequence of *A. vinelandii* DJ (*x*-axis) and the chromosome sequence of *T. turnerae* T7901 (*y*-axis). Only the regions delimited by the positions shown in both axes are shown. The region shared by both corresponds to the major *nif* region, with the modifications noted in the text.

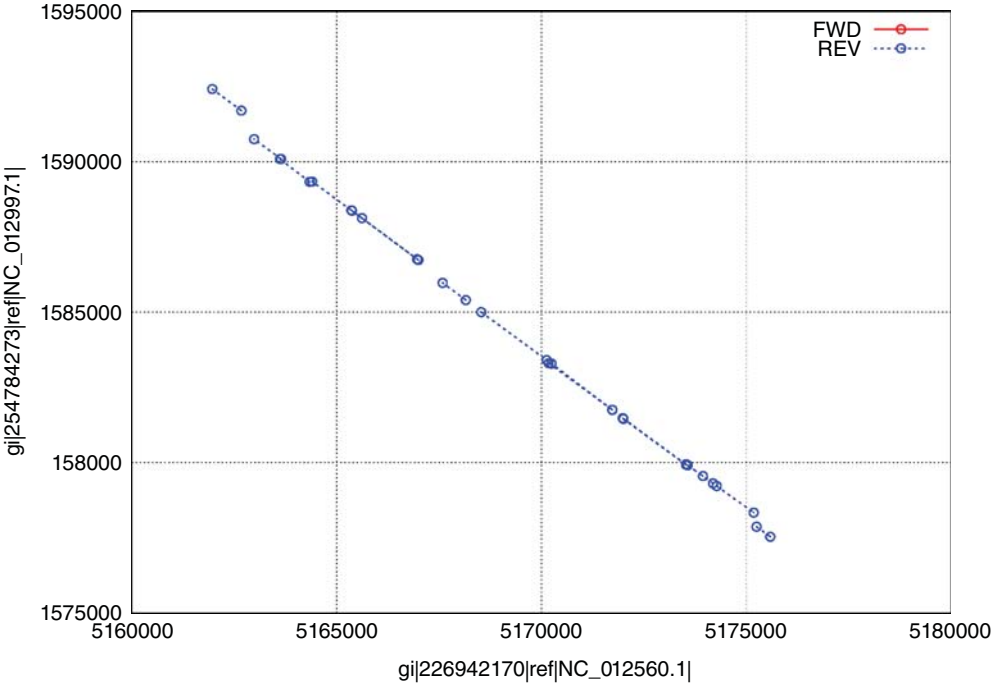

Figure 22.5 Alignment between the chromosome sequence of *A. vinelandii* DJ (*x*-axis) and the chromosome sequence of *T. turnerae* T7901 (*y*-axis). Only the regions delimited by the positions shown in both axes are shown. The region shared by both (which corresponds to Avin_50910 to Avin_51060) includes the minor *nif* region.

Table 22.5 BLAST results Using as queries genes *nifB*, *nifD*, *nifE*, *nifH*, *nifK*, and *nifN* from *A. vinelandii* DJ

	B	D	E	H	K	N
Allochromatium vinosum DSM 180 (gamma)	73/753	84/876	76/756	89/533	79/885	58/542
Azoarcus sp. BH72 (beta)*	72/734	81/861	77/769	87/517	77/874	60/543
Beggiatoa alba B18LD (gamma)	76/780	83/878	80/806	87/525	79/899	63/580
Candidatus Accumulibacter phosphatis clade IIA str. UW-1 (beta)*	72/727	80/842	78/731	86/523	77/872	60/538
Dechloromonas aromatica RCB (beta)*	73/719	81/845	79/765	87/496	75/843	61/560
Methylobacter tundripaludum SV96 (gamma)	75/802	81/872	79/809	86/511	77/878	57/530
Methylomonas methanica MC09 (gamma)	76/783	82/871	81/807	86/508	79/903	59/551
Sideroxydans lithotrophicus ES-1 (beta)*	73/757	82/862	78/778	88/534	78/887	62/569
Teredinibacter turnerae T7901 (gamma)	82/841	86/910	83/827	90/539	82/939	67/653
Thiocapsa marina 5811 (gamma)	74/750	83/882	77/771	89/537	78/887	60/525
Thiocystis violascens DSM 198 (gamma)	75/779	84/892	78/753	89/531	79/892	59/553
Thioflavicoccus mobilis 8321 (gamma)	70/716	82/861	80/779	88/523	76/872	63/555
Thiorhodococcus drewsii AZ1 (gamma)	75/768	84/872	77/791	88/532	80/900	58/537
Thiorhodospira sibirica ATCC 700588 (gamma)	Not found	83/859	77/759	88/532	79/905	60/499
Thiorhodovibrio sp. 970 (gamma)	60/546	81/868	75/756	89/535	76/869	59/547
Thiothrix nivea DSM 5205 (gamma)	76/762	82/874	79/801	89/533	77/872	62/540

The first column contains the organism found (with proteobacterial class in parentheses), and the cells in that row show percent identity/bit score for each hit. For criteria in selecting hits, see the text. Asterisks indicate Betaproteobacteria. BLAST search was done on March 25, 2013.

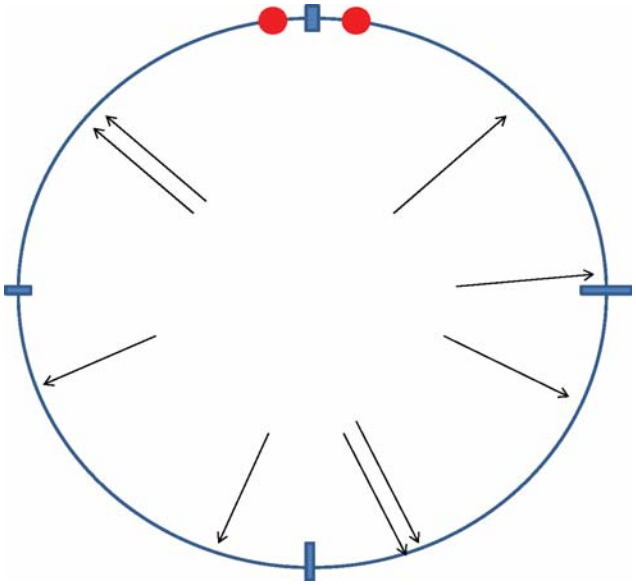

Figure 22.6 Approximate positions of group II introns in the chromosome sequence of *A. vinelandii* DJ (arrows). The two red dots represent the location of the minor (left) and major (right) *nif* regions. At the top is position 1.

and is located from position 3171496 to position 3171619, with two spacers. In addition, the genome contains two cas (CRISPR-associated) groups of protein-coding genes: Avin_17170 to Avin_17240 (spanning region 1,699,773 through 1,707,301 bp) and Avin_31570 to Avin_31630 (spanning region 3,266,532–3,273,695 bp). The second cas operon is thus immediately downstream from the second CRISPR set noted earlier. These data are strong evidence that *A. vinelandii* DJ has a functioning CRISPR-Cas system (CASS).

It has been proposed that a CASS can work as a defensive mechanism against bacteriophage invasion (Makarova et al., 2006). The *A. vinelandii* DJ genome has 40 protein-coding genes annotated with the word "phage." Several of these are scattered throughout the genome and do not form a complete prophage. But 21 of those genes can be found in the region defined by genes Avin_37340 to Avin_37610 (approximately 22 kbp). Part of this region (~5.5 kbp) does match (by MEGABLAST (Altschul et al., 1997)) part of *Pseudomonas aeruginosa* phage phiCTX (Nakayama et al., 1999). But this may be an ancient insertion (the region was not picked up by AH as anomalous), and the whole region may no longer contain a functional prophage. It is a matter however that deserves further investigation.

22.4 CONCLUSION

A. vinelandii is an important model organism for the study of nitrogen fixation and iron–sulfur clusters. The publication of the *A. vinelandii* DJ genome has enabled or facilitated various kinds of studies in these areas. Notable among them was the first transcriptome analysis of *A. vinelandii*, by Hamilton et al. (2011), who studied expression levels of the three nitrogen-fixation systems Nif, Anf, and Vnf that exist in *A. vinelandii* (see also Chapter 9).

Here, results concerning the genome of *A. vinelandii* DJ have been presented, updated, and expanded. Among these, the possible origin of its nitrogen-fixing genes was investigated, which led to the hypothesis that they were inherited

from a common ancestor of *A. vinelandii*, *P. stutzeri*, and *T. turnerae*. Information on the presence of group II introns and the CASS system has also been provided. It is hoped that the results presented here will further facilitate research that depends on the *A. vinelandii* DJ genome.

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Chapter 23

The Genome Sequence of the Novel Rhizobial Species *Microvirga lotononidis* Strain WSM3557^T

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23.1 INTRODUCTION

Microvirga lotononidis WSM3557^T is the type strain of a recently described rhizobial species that is an effective nitrogen (N_2)-fixing microsymbiont of the symbiotically specific African crotalarioid legume *Listia angolensis* (Ardley et al., 2012). We have been studying species of *Listia* (previously *Lotononis* (Boatwright et al., 2011)) because of their potential as pasture legumes in southern Australian agricultural systems. These systems have traditionally relied on biological nitrogen fixation from rhizobial symbioses with introduced legumes, such as medics and subterranean clover, to build up and maintain soil nitrogen for crop and animal production (Howieson et al., 2000; Loi et al., 2005). Since 1975, however, a significant decrease in annual rainfall combined with a decline in its reliability, along with the development of dryland salinity (George et al., 2008), has compromised the effectiveness of annual legumes in these systems. In response, researchers have sought to improve sustainable agricultural productivity by introducing novel perennial pasture legumes (such as *Listia*) and the associated rhizobia that are climatically and edaphically suited to the arid climate and acid, infertile soils of southern Australian farming systems (Howieson et al., 2008).

Listia spp. are water-logging and drought-tolerant form of lupinoid nodules and are infected via epidermal entry, rather than by root hair curling (Ardley et al., 2013; Yates et al., 2007). Most *Listia* species are specifically nodulated by strains of pigmented methylobacteria, but *L. angolensis* forms N_2-fixing nodules only with species of the Gram-negative alphaproteobacterial genus *Microvirga*, a member of the family Methylobacteriaceae (Ardley et al., 2013; Yates et al., 2007). Three of the eight currently described *Microvirga* species are root-nodulating bacteria (RNB) (Ardley et al., 2012). Since this chapter was written, a fourth rhizobial species of *Microvirga, M. vignae*, has been described in Radl et al. (2014).

Nonsymbiotic species of *Microvirga* have been isolated from Australian geothermal waters, a Japanese hot spring, Chinese rice field soil, and Korean atmospheric samples (Kanso and Patel, 2003; Takeda et al., 2004; Weon et al., 2010; Zhang et al., 2009). The rhizobial species *M. lotononidis* and *M. zambiensis* were isolated from *L. angolensis* root nodules collected in Zambia, while *M. texensis* is the specific microsymbiont of the genistoid legume *Lupinus texensis* growing in Texas, USA (Andam and Parker, 2007; Ardley et al., 2012). Additionally, authenticated rhizobial strains subsequently identified as belonging to *Microvirga* have been isolated from nodules of *Phaseolus vulgaris* growing

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in Ethiopia and from northern Australian *Indigofera linifolia* (Ardley et al., 2013; Lafay and Burdon, 2007; Wolde-Meskel et al., 2005). Strains of a novel species of *Microvirga*, with the proposed name of *Microvirga vignae*, are also being developed as rhizobial inoculants for cowpea (*Vigna unguiculata*) in Brazil (Jerri Zilli, pers. comm.). Both *M. lotononidis* WSM3557^T and *M. texensis* Lut6^T have a narrow host range, although WSM3557^T is able to nodulate species of *Leobordea* (previously *Lotononis* (Boatwright et al., 2011)) and *Lotononis* and is effective for N_2 fixation with *Leobordea bolusii*, *L. platycarpa*, and *Lotononis crumanina* (Andam and Parker, 2007; Ardley et al., 2012, 2013).

Most described species of *Microvirga* are either light pink or orange pigmented; absorption spectra of acetone:methanol extracts of *M. lotononidis* WSM3557^T, *M. texensis* Lut6^T, and *M. subterranean* FaiI4^T are consistent with the presence of carotenoids, but not bacteriochlorophyll (Ardley, 2012; Kanso and Patel, 2003). Many are moderately thermophilic and can grow at temperatures of up to 45 °C (Ardley et al., 2012; Kanso and Patel, 2003; Weon et al., 2010). WSM3557^T is fast-growing, forming 0.5–1.5 mm diameter colonies within 2–3 days at the optimum temperature of 41 °C, and can grow at temperatures of up to 45 °C. Colonies are pale pink, opaque, and moderately mucoid on ½ Lupin Agar (½ LA) (Ardley et al., 2012). Lut6^T is also fast-growing, forming dry, pale orange colonies of 0.5–1.5 mm diameter within 2–3 days at the optimum temperature of 39 °C, with a maximum temperature for growth of 43 °C.

Both *M. lotononidis* WSM3557^T and *M. texensis* Lut6^T were selected for sequencing as part of the "Analysis of the clover, pea/bean and lupin microsymbiont genetic pool by studying isolates from distinct Vavilov centres of diversity" Community Sequencing Program (http://genome.jgi.doe.gov/Anaofodiversity/Anaofodiversity.info.html; Joint Genome Institute (JGI) Project IDs: 403024 and 403026, respectively) at the U.S. Department of Energy, JGI. The genome projects for WSM3557^T and Lut6^T are deposited in the Genomes OnLine Database (GOLD) (Liolios et al., 2008), with the accession numbers Gi06493 and Gi06478, respectively. Improved high-quality draft genome sequences are deposited in the Integrated Microbial Genomes (IMG) system (http://img.jgi.doe.gov/cgi-bin/w/main.cgi). *M. lotononidis* WSM3557^T and *M. texensis* Lut6^T have been assigned the IMG Taxon OIDs 2508501114 and 2508501050, respectively.

The basic features of the WSM3557^T genome sequence information and annotation have now been described (Reeve et al., 2014). In presenting our findings to date in this chapter, we have focused on the genomic architecture of WSM3557^T and the presence of genes in this novel microsymbiont that are putatively involved in symbiotic interactions, along with several features that are unusual in rhizobia and found in this genome.

23.2 METHODS

Growth conditions and DNA isolation were according to Reeve et al. (2014); namely, WSM3557^T was grown to mid-logarithmic phase in TY rich media (Reeve et al., 1999) on a gyratory shaker at 28 °C and DNA was isolated from 60 ml of cells using a CTAB (cetyl trimethyl ammonium bromide) bacterial genomic DNA isolation method (http://my.jgi.doe.gov/general/protocols.html).

The draft genome of *Microvirga lotononidis* WSM3557^T was generated at the JGI using a combination of Illumina (Bennett, 2004) and 454 technologies (Margulies et al., 2005). For this genome, we constructed and sequenced an Illumina GAii shotgun library, which generated 71,475,016 reads totaling 5432.1 Mb, and 1 paired-end 454 Titanium library with an average insert size of 10 kb, which generated 582,107 reads totaling 113.9 Mb. All general aspects of library construction and sequencing performed at the JGI can be found at http://www.jgi.doe.gov/. The initial draft assembly contained 444 contigs in 1 scaffold. Illumina sequencing data was assembled with VELVET, version 1.0.13 (Zerbino and Birney, 2008), and the consensus sequence was computationally shredded into 1.5 kb overlapping fake reads (shreds). We integrated Illumina VELVET consensus shreds and the read pairs in the 454 paired-end library using parallel phrap, version SPS – 4.24 (High Performance Software, LLC). The software Consed (Ewing and Green, 1998; Ewing et al., 1998; Gordon et al., 1998) was used in the following finishing process. Illumina data was used to correct potential base errors and increase consensus quality using the software Polisher developed at JGI (Alla Lapidus, unpublished). Possible misassemblies were corrected using gapResolution (Cliff Han, unpublished), Dupfinisher (Han and Chain, 2006), or sequencing cloned bridging polymerase chain reaction (PCR) fragments with subcloning. Gaps between contigs were closed by editing in Consed, by PCR, and by Bubble PCR (J-F Cheng, unpublished) primer walks. A total of 303 additional reactions were necessary to close gaps and to raise the quality of the finished sequence. The estimated genome size is 7.2 Mb, and the final assembly is based on 59.7 Mb of 454 draft data that provides an average 8.3× coverage of the genome and 2160 Mb of Illumina draft data, which provides an average 300× coverage of the genome.

The methods for genome annotation are detailed in Reeve et al. (2014). Specifically, genes were identified using Prodigal (Hyatt et al., 2010) and annotated using the IMG annotation pipeline (Mavromatis et al., 2009). The predicted coding sequences (CDSs) were translated and used to search the National Center for Biotechnology Information (NCBI) nonredundant database, UniProt, TIGRFam, Pfam, PRIAM, Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Group (COG), and InterPro databases. These data sources were combined to assert a product description for each predicted protein.

Noncoding genes and miscellaneous features were predicted using tRNAscan-SE (Lowe and Eddy, 1997), RNAMMer (Lagesen et al., 2007), Rfam (Griffiths-Jones et al., 2003), TMHMM (Krogh et al., 2001), and SignalP (Bendtsen et al., 2004). Additional gene prediction analyses and functional annotation were performed within the IMG (Expert Review) platform (http://img.jgi.doe.gov/er) (Markowitz et al., 2009). Further examination of the WSM3557^T genome used the analysis tools provided within the IMG platform (http://img.jgi.doe.gov/cgi-bin/w/main.cgi).

Plasmid profiling was performed using a modified Eckhardt procedure (Priefer, 1984).

23.3 RESULTS AND DISCUSSION

23.3.1 Structural Organization of the WSM3557^T Genome

The improved high-quality draft genome of WSM3557^T consists of 7,082,538 bp, with 63.08% G + C content, and is composed of 18 scaffolds of 104 contigs (Reeve et al., 2014). The genome is intermediate in size for root nodule bacteria, being smaller than *Bradyrhizobium japonicum* USDA 110 (9.105 Mbp) (Kaneko et al., 2002) and the *Methylobacterium* strains *M. nodulans* ORS 2060^T (8.839 Mbp) and *M.* sp. 4-46 (7.737 Mbp) (Marx et al., 2012), but larger than *Ensifer* (*Sinorhizobium*) *medicae* WSM419 (6.817 Mbp) (Reeve et al., 2010), *Rhizobium etli* CFN42 (6.53 Mbp) (González et al., 2006), and *Azorhizobium caulinodans* ORS 571 (5.37 Mbp) (Lee et al., 2008). From a total of 7036 genes, 6952 were protein encoding and 84 were RNA-only encoding genes (Reeve et al., 2014). There is one rRNA operon, with one full-length and one partial 23S rRNA gene and three partial 5S rRNA genes. The rRNA operon is on scaffold MLG.10 and includes genes for three tRNAs (tRNA-ala, tRNA-ile, and tRNA-met). There are 53 additional tRNA genes and the total represents 45 tRNA species. WSM3557^T is a selenocysteine synthesizer, possessing genes that encode seryl-tRNA synthetase (MicloDRAFT_00050960), selenophosphate synthase (MicloDRAFT_00024690), and seryl-tRNA (Sec) selenium transferase (MicloDRAFT_00024720). The distribution of genes into COGs functional categories is presented in Table 23.1. The number and percentage of genes assigned to each COG functional category is similar to that found in other sequenced

Table 23.1 Number of protein-coding genes of *Microvirga* sp. WSM3557^T associated with the general COG functional categories

Code	COG Category	Gene Count	% of Total [6170]
E	Amino acid transport metabolism	612	10.76
J	Carbohydrate transport and metabolism	436	7.66
D	Cell cycle control, mitosis, and meiosis	38	0.67
N	Cell motility	76	1.34
M	Cell wall/membrane biogenesis	254	4.46
L	Chromatin structure and dynamics	7	0.12
H	Coenzyme transport and metabolism	193	3.39
Z	Cytoskeleton	0	0.00
V	Defense mechanisms	72	1.27
C	Energy production conversion	307	5.40
W	Extracellular structures	1	0.02
S	Function unknown	586	10.30
R	General function prediction only	678	11.92
P	Inorganic ion transport and metabolism	319	5.61
U	Intracellular trafficking and secretion	73	1.28
I	Lipid transport and metabolism	179	3.15
F	Nucleotide transport and metabolism	105	1.85
Y	Nuclear structure	0	0.00
O	Posttranslational modification, protein turnover, chaperones	178	3.13
K	Replication, recombination, and repair	431	7.57
A	RNA processing and modification	1	0.02
Q	Secondary metabolite biosynthesis, transport, and catabolism	171	3.01
T	Signal transduction mechanisms	375	6.59
L	Transcription	398	6.99
G	Translation, ribosomal, structure, and biogenesis	200	3.51
-	Not in COGs	1919	27.27

From Reeve et al. (2014) with permission.

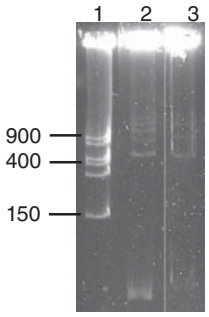

Figure 23.1 Eckhardt gel plasmid profiles of rhizobial *Microvirga* strains. The *Rhizobium leguminosarum* strain VF39 was used as a marker. The size of specific VF39 plasmids (kb) is shown. Lane 1, VF39; lane 2, Lut6^T; lane 3, WSM3557^T.

rhizobial strains, although a comparatively large number of genes (431; 7.57%) are associated with replication, recombination, and repair.

The plasmid profile of WSM3557^T shows that it possesses a multipartite genome architecture. Both WSM3557^T and *M. lupini* Lut6^T have multiple replicons (Fig. 23.1).

According to the gel image, WSM3557^T contains four plasmids, ranging in size from greater than 900 Kbp to less than 150 Kbp. Lut6^T harbors six plasmids, including three that are greater than 900 Kbp in size.

This number of plasmids in WSM3557^T is congruent with the presence of four copies of each of the *repABC* genes, which are present on large, low copy-number plasmids and on some secondary chromosomes in a number of alphaproteobacterial genera, and are responsible for the replication and segregation properties of these replicons (Cervantes-Rivera et al., 2011). In scaffold MLG.13, *repABC* are present in a cluster (MicloDRAFT_00032150–MicloDRAFT_00032180), together with a gene encoding the antisense RNA that modulates *repC* expression (Venkova-Canova et al., 2004). The presence of large plasmids in WSM3557^T and Lut6^T makes them notably different from the closely related and comparatively slow-growing genera *Bradyrhizobium* and *Methylobacterium*, which have large chromosomes, with small plasmids present in some strains (Cytryn et al., 2008; Marx et al., 2012).

A detailed analysis of the different replicons of WSM3557^T and Lut6^T would require their genomic structures to be fully resolved before they can be aligned and compared. However, a dot plot analysis of the assembled scaffolds of WSM3557^T and Lut6^T, using MUMmer (Kurtz et al., 2004), revealed considerable disruptions of synteny, indicating a high level of evolutionary divergence between the two strains. Dot plots of WSM3557^T mapped to the complete genome sequences of *Bradyrhizobium japonicum* USDA 110 and *Methylobacterium nodulans* ORS 2060^T showed no synteny.

23.3.2 Symbiotic Loci

For all known legume–rhizobia associations except that of *Aeschynomene* spp. and particular strains of *Bradyrhizobium* (Bonaldi et al., 2011), symbiosis is initiated when plant-derived flavonoids induce expression of rhizobial nodulation (*nod*, *nol*, and *noe*) genes and the subsequent synthesis of lipochito-oligosaccharide (LCO) Nod factors (see Chapters 50, 51). Regulation of *nod* gene expression is mediated by the transcriptional regulator NodD, the LCO backbone is encoded by the canonical *nodABC* genes, and other nodulation loci encode substituent groups that decorate the LCO core (Perret et al., 2000). The Nod factors induce plant genes that control and coordinate the processes of bacterial infection and nodule organogenesis (Oldroyd et al., 2011). Within the nodule, the rhizobia differentiate into N_2-fixing bacteroids and transcription of the nitrogen fixation (*nif* and *fix*) genes is induced (Terpolilli et al., 2012; see Sections 9–12).

Although the molecular basis of the dialog between *L. angolensis* and *M. lotononidis* has yet to be determined, the WSM3557^T genome possesses genes encoding synthesis of LCO structures and substituent groups, suggesting that a Nod factor is required in the establishment of this symbiosis. Genome regions containing symbiotic loci have a mosaic structure, with a large number of transposases, integrases, and inactivated derivatives present. Many of the genes required for nodulation and nitrogen fixation are clustered on the scaffold MLG.4 (146,360 bp, G + C = 0.62%).

23.3.3 Nodulation Genes

The gene neighborhood of WSM3557^T nodulation loci harbored on the MLG.4 scaffold is shown in Figure 23.2.

Organization of the *nod* cluster is similar to that seen in *Methylobacterium nodulans* ORS 2060^T, which specifically nodulates some species of *Crotalaria* (Sy et al., 2001), and the pigmented *Methylobacterium* sp. strains 4-46 and WSM2598, which are microsymbionts of *Listia bainesii* (Ardley et al., 2013; Fleischman and Kramer, 1998; Yates et al., 2007). These strains and other sequenced strains that share high sequence similarity with WSM3557^T *nod* genes are included for comparison in Figure 23.2.

The WSM3557^T genome contains one copy of the transcriptional regulator *nodD* (MicloDRAFT_00005370), which is upstream and transcribed in the opposite direction to a *nodYABC* operon (MicloDRAFT_00005280–MicloDRAFT_00005310). The NodD protein shares highest identity with NodD of rhizobial South African *Burkholderia* strains. No other homologs of *nodD* have been found in the WSM3557^T genome. WSM3557^T *nodY* is homologous with *Bradyrhizobium nodY*, which encodes a signal transduction response regulator and until now has been found exclusively in bradyrhizobia (Menna and

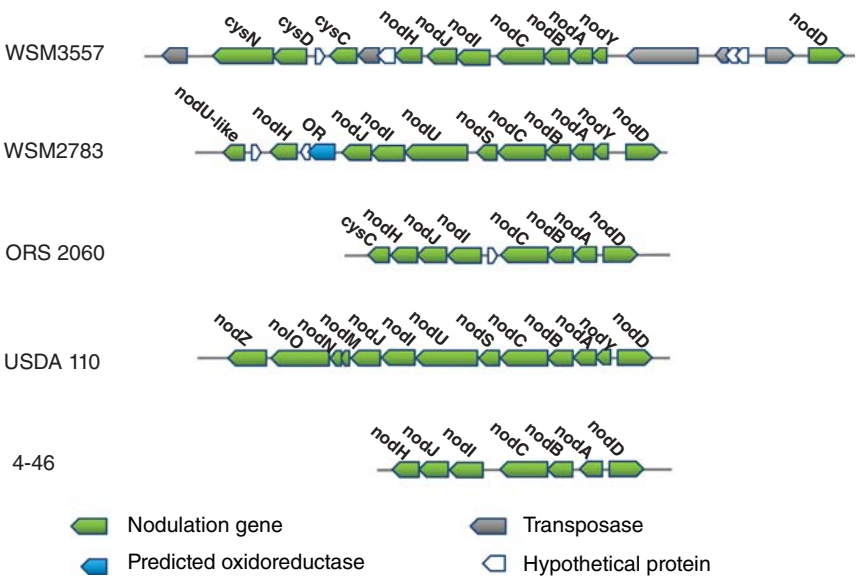

Figure 23.2 Physical map of nodulation loci of the sequenced genomes *Microvirga lotononidis* WSM3557^T, *Methylobacterium nodulans* ORS 2060, *Methylobacterium* sp. 4-46, *Bradyrhizobium japonicum* USDA 110, and *Bradyrhizobium* sp. WSM2783. Arrows show the direction of transcription.

Hungria, 2011). The highest identity (55%) is with NodY of *Bradyrhizobium* sp. WSM2783, which nodulates species of *Leobordea* and *Lotononis* (Ardley et al., 2013). NodA of WSM3557^T clusters with that of other rhizobia that nodulate African genistoid legumes, sharing 85%, 84%, and 81% identity with NodA of *Bradyrhizobium* sp. WSM2783, *Burkholderia* sp. STM678 (which nodulates species in tribe Podalyrieae from the South African Cape Fynbos biome (Gyaneshwar et al., 2011)), and *M. nodulans* ORS 2060^T, respectively. The WSM3557^T NodB has highest identity (71%) with *Bradyrhizobium* sp. WSM2783, while NodC shares highest identity (81%) with *Sinorhizobium fredii* HH103.

Immediately downstream of *nodYABC* are *nodIJ*, *nodH*, a gene encoding a protein of unknown function, *cysC, cysDN,* and genes encoding transposases (MicloDRAFT_00005190–MicloDRAFT_00005270). NodI and NodJ share highest identity of 87.5% and 81.6%, respectively, with *Rhizobium giardinii* bv. *giardinii* H152^T. The highest identities for WSM3557^T NodH are 83%, 80.2%, and 76.2% with *Bradyrhizobium* sp. WSM2783, *Microvirga texensis* Lut6^T, and *M. nodulans* ORS 2060^T NodH, respectively. The open reading frame (ORF) immediately downstream of *nodH* encodes a hypothetical protein with no homology to any known bacterial proteins. BLASTP analysis gave less than 30% identity with proteins from all organisms in the NCBI database. WSM3557^T CysC shares highest identity (59.3%) with *Hyphomicrobium* sp. MC1, while CysD and CysN share highest identity (83.5% and 61.4%, respectively) with *Methylobacterium* sp. strains 4-46 and WSM2598. In the methylobacterial strains, *cysDN* (and *cysC* in 4-46 and WSM2598) are not grouped in the nodulation gene cluster, but found elsewhere in the genome. The *cysCDN* genes, along with *nodH*, encode products required for Nod factor sulfation. *cysDNC* are homologs of *nodPQ* and encode subunits of adenosine triphosphate (ATP) sulfurylase (CysDN) and adenosine 5′-phosphosulfate (APS) kinase (CysC), which associate into a multifunctional protein complex and catalyze formation of an activated sulfate group (Schwedock et al., 1994). The activated sulfate group is then transferred to the Nod factor by the sulfotransferase NodH (Schultze et al., 1995). The Nod factors produced by WSM3557^T and the pigmented methylobacteria have not yet been characterized. However, the Nod factor structures of ORS 2060^T were identified as chitopentamers containing a $C_{18:1}$ or $C_{16:0}$ fatty acyl chain and sulfated at the reducing end (Renier et al., 2008). From their similarity with ORS 2060^T *nod* genes, it is reasonable to assume that the *nod* genes of WSM3557^T, 4-46, and WSM2598 also encode sulfated Nod factors, which may be required for nodulation of *Listia* species.

23.3.4 Genes Required for Nitrogen Fixation

The WSM3557^T MLG.4 scaffold also contains loci that are homologs of genes required for nitrogen fixation in other rhizobia. These are organized into two gene clusters. The first containing *nifA-nifB-fdxN-nifZT* (MicloDRAFT_00006160–MicloDRAFT_00006200) is 74,979 bp downstream of *nodD*. The second cluster is 10,434 bp downstream of *nifT* and contains *nifHDKENX1X2-fdxB* (MicloDRAFT_00006320–MicloDRAFT_00006400). The encoded proteins all share high identity with homologs found

in *M. lupini* Lut6^T (>91%) and in *Rhizobium mesoamericanum* STM6155 and various sequenced *Sinorhizobium* and *Mesorhizobium* strains (>76%). Another cluster of *nif/fix* genes is located on the scaffold MLG.1, and contains the *fixABCX* operon and an operon consisting of a gene encoding a scaffold protein (of the HesB/IscA family) for iron-sulfur cluster assembly and *nifSV-cysE-nifW* (MicloDRAFT_00000030–MicloDRAFT_00000110). Genes in this cluster also share high identity [>80%] with homologs in *M. lupini* Lut6^T and *Sinorhizobium* and *Mesorhizobium* strains. Scaffold MLG.9 contains a homolog of *nifU* (MicloDRAFT_00017310), along with the *fixNOQP-fixGHIS* and *fixKLJ* clusters (MicloDRAFT_00022650–MicloDRAFT_00022770) that are essential for microoxic respiration (Kaneko et al., 2011). The encoded MLG.9 nitrogen-fixation proteins share highest identity with homologs found in *M. lupini* Lut6^T (72.9–93.8%) or in strains of *Azorhizobium, Bradyrhiz*obium, *Methylobacterium*, *Starkeya*, and *Xanthobacter* (53.3–81.4%).

The alternative sigma factor RpoN is essential for N_2 fixation (Fischer, 1994). The WSM3557^T genome has two copies of the *rpoN* gene. *rpoN1* (MicloDRAFT_00017570) is found in scaffold MLG.9 between the *nifU* and *fixN* genes, while *rpoN2* (MicloDRAFT_00070370) is clustered with *nifQ* (MicloDRAFT_00070390) in scaffold MLG.17. *Rhizobium etli* strain CNPAF512 also contains two copies of *rpoN*; *rpoN1* is required for housekeeping functions, while *rpoN2* is essential for symbiotic nitrogen fixation (Michiels et al., 1998). Similar differential regulation of the two *rpoN* gene copies may occur in WSM3557^T. This is supported by the different phylogenies of the *Microvirga* WSM3557^T and Lut6^T *rpoN* copies. The *rpoN1* gene of both WSM3557^T and Lut6^T has highest identity (54.7–57.3%) with strains of methylobacteria and with *Azorhizobium doebereinerae* UFLA1-100. In contrast, *rpoN2* of Lut6^T (which in this genome is clustered with other nitrogen-fixation genes on scaffold MLH112) and *rpoN2* of WSM3557^T have highest identity (77.2–80.3%) with strains of *Mesorhizobium* and *Sinorhizobium.* There also appears to be disparate phylogenies between the WSM3557^T *nif/fixABCX* genes present on the MLG.1, 4, and 17 scaffolds and the *fix* genes required for microoxic respiration that are on the MLG.9 scaffold. The *nif* genes are involved in the synthesis, assembly, and functioning of the nitrogenase complex (Rubio and Ludden, 2008), while the *fixABCX* genes are postulated to encode a product that could facilitate the transfer of electrons to nitrogenase (Earl et al., 1987; Fischer, 1994; Scott and Ludwig, 2004). The close affinity of the MLG.1, 4, and 17 *nif* and *fix* genes to those of *Sinorhizobium* and *Mesorhizobium* suggests that these genes were acquired through horizontal transfer.

23.3.5 Additional Genes Required for Symbiosis Are Present on Scaffold MLG.4

Other loci putatively involved in symbiosis, such as the *dct* and *groESL* genes, have been identified on the *M. lotononidis* WSM3557^T scaffold MLG.4.

The *dctA* gene encodes a C4-dicarboxylate transport protein responsible for transport of host plant-supplied carbon to bacteroids and is required for symbiotic nitrogen fixation in rhizobia, while *dctB* and *dctD* encode a two-component regulatory system that regulates expression of DctA; homologs of these genes appear to be ubiquitous in rhizobia (Ronson et al., 1984; Yurgel and Kahn, 2004). In WSM3557^T, *dctA* and the adjacent divergently transcribed *dctBD* (MicloDRAFT_00005410–MicloDRAFT_00005430) are located in close proximity to *nodD*. Highest identity of the WSM3557^T DctA, B, and D (78.6–85.1%) is to homologs from strains of *Mesorhizobium loti.* Two additional copies of *dctA* (MicloDRAFT_00022000, MicloDRAFT_00061080) have been identified in the WSM3557^T genome, however, neither of these is associated with the corresponding two-component regulatory system *dctBD* and they share 57.24% and 49.12% identity, respectively, with the MLG.4 DctA.

GroEL chaperonins are required for the formation of a functional nitrogenase in *Bradyrhizobium japonicum* (Fischer et al., 1999). There are five pairs of chaperonin *groES-groEL* genes present in the WSM3557^T genome; one pair (MicloDRAFT_00005680–MicloDRAFT_00005670) is clustered with other symbiotic loci on MLG.4 indicating its potential role in symbiosis, while the other pairs (MicloDRAFT_00011830–MicloDRAFT_00011840; MicloDRAFT_00043640–MicloDRAFT_00043630; MicloDRAFT_00008770–MicloDRAFT_00008780; MicloDRAFT_00052250–MicloDRAFT_00052240) are on separate scaffolds.

23.3.6 Secretion Systems

23.3.6.1 Type I Secretion System. Type I secretion system (T1SS) genes are present in most rhizobia (Schmeisser et al., 2009). The secretion machinery consists of three proteins localized in the cell envelope: a specific outer membrane protein (OMP) and two cytoplasmic membrane proteins, one is an ATP-binding cassette (ABC) transporter protein and the other is a membrane fusion adaptor protein (Delepelaire, 2004). A number of T1SS homologs have been identified in WSM3557^T, including one TolC-like OMP (MicloDRAFT_00054540) and six copies each of PrtD family ABC transporter proteins and HlyD/PrtE family membrane fusion adaptor proteins that occur as pairs (MicloDRAFT_00007510/MicloDRAFT_00007520; MicloDRAFT_00037970/MicloDRAFT_00037980; MicloDRAFT_00053240/MicloDRAFT_00053250; MicloDRA

FT_00054970/MicloDRAFT_00054980; MicloDRAFT_00055180/MicloDRAFT_00055190; MicloDRAFT_00057510/MicloDRAFT_00057520). Proteins secreted by the T1SS vary greatly in size (Delepelaire, 2004) and include repeats-in-toxin (RTX) exoproteins, a family of proteins with diverse biological functions, which can act as virulence factors (Linhartová et al., 2010). A variable number of genes encoding RTX toxins and related Ca^{2+}-binding proteins (COG2931) are found in sequenced rhizobial genomes in the JGI database. They are not present in *Azorhizobium caulinodans* ORS 571^T or *A. doebereinerae* UFLA1-100^T, or in any rhizobial *Burkholderia* strains. *Bradyrhizobium*, *Cupriavidus taiwanensis*, *Mesorhizobium*, *Methylobacterium*, *Rhizobium*, and *Sinorhizobium* strains have from 1 to 10 *rtx* genes. In contrast, the *Microvirga* strains possess a remarkably large number of genes encoding RTX toxins and the related Ca^{2+}-binding proteins: 36 and 58, respectively, for WSM3557^T and Lut6^T.

23.3.6.2 Type II Secretion Systems. Although the type II general secretion pathway (Sandkvist, 2001) is absent in WSM3557^T and Lut6^T, both the general export pathway (GEP Sec) and the twin arginine translocation (TAT) pathway are present in both strains.

23.3.6.3 Type III Secretion System. Type III secretion systems (T3SSs) are used by plant and animal pathogens to translocate bacterial proteins into host cells where they mediate cellular functions, often to suppress host immune responses (Jones and Dangl, 2006). Rhizobial T3SS loci were first identified in the *Sinorhizobium fredii* strain NGR234 (Freiberg et al., 1997) and have since been found in strains of *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Rhizobium etli* (Deakin and Broughton, 2009). They are symbiotically active and have been shown to modulate nodulation in host plants, either positively or negatively (Krause et al., 2002; Marie et al., 2003; Okazaki et al., 2010).

Gene clusters encoding T3SS components are found in both WSM3557^T and Lut6^T. In WSM3557^T, T3SS loci are found in scaffolds MLG.2 and MLG.12. Homologs of *ttsI*, which encodes a response regulator (Marie et al., 2004), and *rhcC2*, which encodes an OMP of the secretion machinery (Schmeisser et al., 2009), are located on MLG.12 (MicloDRAFT_00026870 and MicloDRAFT_00026880). The remaining T3SS gene cluster (MicloDRAFT_00002700–MicloDRAFT_00002940), encoding essential components of the secretion apparatus and secreted proteins, is located on the small (26,102 bp) scaffold MLG.2. The organization of the gene clusters is similar to that of the T3SS loci found in the symbiotic plasmid of NGR234 (Schmeisser et al., 2009) and in *Mesorhizobium loti* MAFF30399 (Sánchez et al., 2009), except that *nolU* is absent (Fig. 23.3).

23.3.6.4 Type IV Secretion System. Type IV secretion systems (T4SSs) are ancestrally related to bacterial conjugation machines and mediate the transfer of DNA and protein substrates to phylogenetically diverse prokaryotic and eukaryotic target cells (Christie et al., 2005). The paradigmatic *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*) T4SS machine is formed by the 12 VirBD4 proteins (Christie, 2004). Both *M. lotononidis* WSM3557^T and *M. texensis* Lut6^T possess *virBD4* loci. In WSM3557^T, these are found on scaffold MLG.13 in an operon (MicloDRAFT_00030620–MicloDRAFT_00030730) that is highly similar to the gene arrangement of the T4SS loci harbored in plasmid pAtK84c of *Rhizobium rhizogenes* strain K84 and plasmid pMLa of *M. loti*

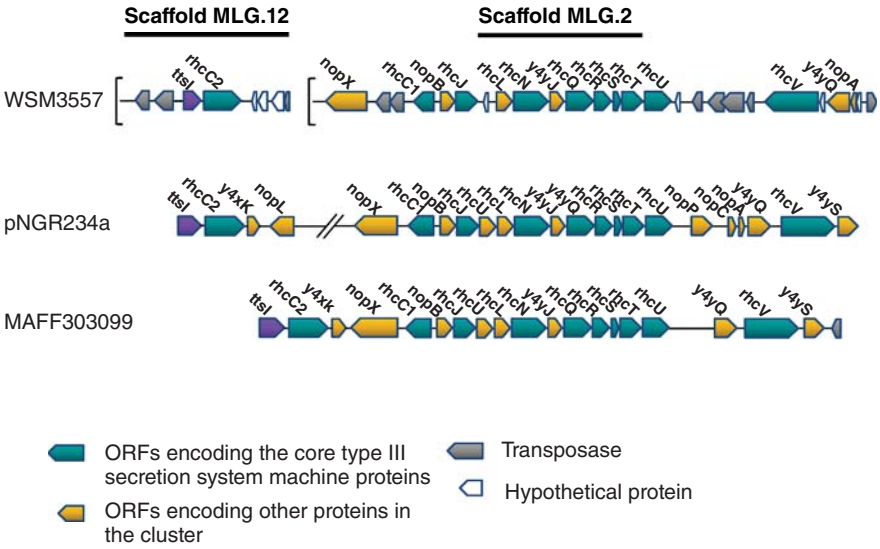

Figure 23.3 Type III secretion system loci of *Microvirga lotononidis* WSM3557^T, *Mesorhizobium loti* MAFF30399, and *Sinorhizobium fredii* NGR234.

MAFF30399. Two genes encoding hypothetical proteins (MicloDRAFT_00030680 and MicloDRAFT_00030690) are within this operon. *virB5* (MicloDRAFT_00030770) is found in an adjacent, divergently transcribed operon. WSM3557^T lacks *virB7*, which in *R. radiobacter* encodes an outer membrane T-DNA transfer lipoprotein required for T-pilus biogenesis (Christie et al., 2005). WSM3557^T VirBD4 shares highest identity with homologs in Lut6^T (78.7–95%) and in *R. rhizogenes* K84 and *Methylocystis* sp. Rockwell ATCC 49242 (39.8–65.9%). Genes encoding VirD (other than VirD4) and VirE are not present.

In *A. tumefaciens,* the virulence protein homologs VirJ/AcvB have been shown to be involved in the T4SS (Pan et al., 1995; Pantoja et al., 2002). *M. lotononidis* WSM3557^T scaffold MLG.13 also contains the *acvB* ortholog MicloDRAFT_00044170, located downstream of MicloDRAFT_00044180, an ortholog of the *Sinorhizobium medicae* WSM419 low pH-inducible gene *lpiA* (Reeve et al., 2006). The AcvB and LpiA proteins have been implicated in the modification of membrane lipid structure (Reeve et al., 2006; Sohlenkamp et al., 2007). Analysis of sequenced bacterial genomes reveals that the *acvB/lpiA* operon is highly conserved in Gram-negative pathogens and symbionts, suggesting the importance of these proteins in prokaryotic/eukaryotic interactions.

23.4 CONCLUSIONS

Rhizobial species of *Microvirga* constitute a novel group of nitrogen-fixing legume-symbiotic Alphaproteobacteria. *Microvirga* strains are uncommon microsymbionts of legumes, yet *M. lotononidis* WSM3557^T and *M. texensis* Lut6^T form highly specific and effective N_2-fixing symbioses with *Listia angolensis* and *Lupinus texensis*, respectively. Our studies have shown that the *M. lotononidis – Listia angolensis* symbiosis is an example of symbiotic specificity in an epidermally infected legume; however, the molecular basis of this specificity has not yet been determined.

Analysis of the sequenced genome of WSM3557^T suggests that this strain has acquired symbiotic loci by horizontal gene transfer from rhizobial donor strains. Symbiotic loci in WSM3557^T that show evidence of horizontal gene transfer include genes required for C4-dicarboxylate transport, the synthesis, assembly, and functioning of the molybdenum nitrogenase complex and Nod factor synthesis. Nod factor proteins have highest similarity with *Bradyrhizobium*, *Methylobacterium*, and *Sinorhizobium* Nod proteins. The FixNOPQ proteins required for microoxic respiration are most closely related to those of *Azorhizobium*. In contrast, other Nif, Fix, and Dct proteins appear to be most similar to those of *Mesorhizobium* and *Sinorhizobium*. WSM3557^T also appears to have acquired a T3SS from a mesorhizobial species. Whether the WSM3557^T T3SS has a role in modulating legume symbiosis remains to be determined. Likewise, the role of the large number of predicted RTX-like calcium-binding secreted proteins has not yet been examined.

Future studies of WSM3557^T will include functional analyses of the genes and regulons important to symbiotic performance and saprophytic competence. Exploitation of the genome sequence of WSM3557^T will be pivotal to gaining an understanding of the symbiotic process in symbiotically specific epidermally infected legumes. The proposed research is expected to deliver significant new information on legumes and rhizobia that are new to agriculture, new to science, and predicted to be important components of environmentally sustainable agriculture.

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Chapter 24

Genome Characteristics of *Frankia* sp. Reflect Host Range and Host Plant Biogeography

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24.1 INTRODUCTION

An actinobacterium from the genus *Frankia* was first isolated in 1978 (Callaham et al., 1978) despite hundreds of prior attempts that failed, in hindsight, because of the slow growth rate of the organisms, presence of fast-growing contaminants in plant tissues, and the fact some lineages have evolved toward obligate symbiosis (Baker and Torrey, 1979). In addition, some of the genomes that have been sequenced have a large number of active genetic elements that may confer instability (Bickhart et al., 2009). For the same reasons, genetic transformation systems have not been developed despite numerous attempts using regenerated protoplasts (Normand et al., 1987; Tisa and Ensign, 1987) or electroporation (Cournoyer and Normand, 1992; Myers and Tisa, 2003). Attempts using complementation of *Rhizobium nodB* and *nodC* mutants with *Frankia* DNA banks in cosmid vectors have also failed (Ceremonie et al., 1998).

Apart from obvious basic physiological processes, including nitrogen fixation, hydrogen uptake, and bacteriohopane biosynthesis, bacterial symbiotic determinants are not known so far, even though the infection process and its ultimate outcome bear many similarities with those of the rhizobia (*sensu lato*) in legumes. Nodule establishment starts with either a deformed root hair entry or an intercellular penetration, followed by an infection thread, induction at a distance of cell differentiation, and emergence of a nodule invaded by frankial cells that differentiate into specialized forms (Benson and Silvester, 1993). The symbiotic program of host plants *Alnus* and *Casuarina* has been monitored by an expressed-sequence-tag (EST) approach and found to involve most of the determinants known in legumes (Hocher et al., 2011), with a special mention for SymRK that was found necessary for triggering infection by *Frankia* (Gherbi et al., 2008; see also Chapters 42, 43, 55).

The host plant symbiotic lock is thus beginning to be known; however, the identity of the bacterial symbiotic key remains elusive. Although some work has been done to identify the compound(s) that deform root hairs in *Alnus* (Van Ghelue et al., 1997; Ceremonie et al., 1999), they can only be postulated to be small hydrophilic molecules (below 2000 Da) that resists autoclaving and the chitinases tested.

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Table 24.1 Characteristics of genomes of genus *Frankia*

Trait	Fd *Frankia* Symbiont of Datisca glomerata (Dg)	Fc *Frankia* sp. Strain CcI3	Fa *Frankia alni* Strain ACN1 4a	QA3 *Frankia* sp. Strain QA3	Fe *Frankia* sp. Strain EAN1p ec	EUN1f *Frankia* sp. Strain EUN1f	BCU110501 *Frankia* sp. Strain BCU11 0501	BMG5.12 *Frankia* sp. Strain BMG5. 12	EuI1c *Frankia* sp. Strain EuI1c	DC12 *Frankia* sp. Strain DC12	CN3 *Frankia* sp. Strain CN3
Cluster	II	Ib	Ia	Ia	III	III	III	III	III	IV	IV
Genome size (nt)	5,323,336	5,433,628	7,497,934	7,590,853	8,982,042	9,322,173	7,891,711	7,589,313	8,815,781	6,884,336	9,978,592
G+C%	70.04	70.08	72.83	72.56	71.15	72.31	72.39	71.67	70.82	71.93	71.81
No. of genes	4,579	4,621	6,795	6,546	7,250	7,833	6,839	6,342	7,264	5,858	8,412
No. of tRNA	45	46	46	46	47	47	47	51	46	46	68
No. of rRNA operons	2	2	2	2	3	3	2	2	3	3	2
Protein-coding density %	78.15	84.94	86.31	82.04	83.71	84.06	84.66	85.50	86.10	84.44	83.73
Genome accession number	CP002801	CP000249.1	CT573213	CM001489.1	CP000820.1	NC_014666	—	—	ADGX00000000	—	AGJN00000000
Symbiotic status	**Obligately symbiotic**	**Facultative limited**	**Facultative symbiont**	**Facultative symbiont**	**Facultative symbiont**	**Facultative symbiont**	**Facultative symbiont**	**Facultative symbiont**	**Asymbiotic/ defective**	**Asymbiotic/ defective**	**Asymbiotic/ defective**
Geographic distribution	Tropical and temperate inside host plants	Tropical inside host plants or in proximity to host in soil	Circumpolar, temperate, and subtropical mountains (soils)	Circumpolar, temperate, and subtropical mountains (soils)	Global (soils)	Global (soils)	Global (soils)	Global (soils)	Global (soils)	?	?
Host plants	Rosaceae Rhamnaceae (N. Amer.) Coriariaceae Datiscaceae	Casuarinaceae Myricaceae	Betulaceae Myricaceae	Betulaceae Myricaceae	Elaeagnaceae Gymnostoma Rhamnaceae (S. Amer.) Myricaceae	Elaeagnaceae Gymnostoma Rhamnaceae (S. Amer.) Myricaceae	Elaeagnaceae Gymnostoma Rhamnaceae (S. Amer.) Myricaceae	Elaeagnaceae Gymnostoma Rhamnaceae (S. Amer.) Myricaceae	Elaeagnaceae Gymnostoma Rhamnaceae (S. Amer.) Myricaceae	None	None

In addition, some genomic and proteomic work has shown that frankiae devote a considerably smaller portion of their genome to making secreted proteins than do other bacteria, including polysaccharide hydrolases and other hydrolases (Mastronunzio et al., 2009). This observation has led to the hypothesis that the frankial symbiosis proceeds partly by avoiding triggering plant defense responses during infection (Mastronunzio et al., 2009).

On the basis of 16S rRNA sequencing, four clusters of *Frankia* strains have been recognized: (i) those infective on members of the Betulaceae (*Alnus*), Casuarinaceae (*Casuarina*, *Allocasuarina*), and Myricaceae (*Morella*, *Myrica*); (ii) those present in nodules of members of the Rosaceae (*Cercocarpus*, *Chamaebatia*, *Dryas*, *Purshia*), Datiscaceae (*Datisca*), Coriariaceae (*Coriaria*), and the North American *Ceanothus* (Rhamnaceae) that have not been grown in pure culture; (iii) those infective on members of the Elaeagnaceae (*Elaeagnus*, *Hippophae*, *Shepherdia*), *Gymnostoma*, and certain southern hemisphere Rhamnaceae (*Colletia*, *Discaria*, *Talguenia*, *Trevoa*); and (iv) strains of noninfective or noneffective (defective) saprophytes (Normand et al., 1996).

The different *Frankia* strain groups have contrasted lifestyles that range from obligate symbiotic to facultatively symbiotic to nonsymbiotic. **Obligate** symbionts have resisted, so far, all attempts at cultivation in pure culture and are apparently absent from soils that are devoid of their host plants; this is the case for the cluster 2 strains tested so far, of many *Casuarina*-infective and *Allocasuarina*-infective cluster 1b strains and cluster 1a *Alnus*-infective strains that produce many sporangia in nodules called Sp + (Simonet et al., 1999; Vanden Heuvel et al., 2004). Some other lineages are **facultative/limited**. They contain some strains that can be cultivated *in vitro* yet are not detected by trapping from soils lacking their host plants, and have several phylogenetic neighbors that resist attempts at growth in pure culture. This is the case for *Casuarina*-infective and *Allocasuarina*-infective cluster 1b strains (Diem et al., 1983; Paschke and Dawson, 1993). Other strains are **facultative symbionts** that can be cultivated readily and grown in soils in the absence of their host plants; this is the case of *Alnus*-infective cluster 1a strains (Berry and Torrey, 1979; Callaham et al., 1978). Other facultative strains that are abundant in soils in the absence of their normal hosts can sometimes be recovered from plants with which they do not usually form symbiotic nodules have been designated atypical; this is the case of cluster 3 strains (Gauthier et al., 1981). Finally, some strains are **asymbiotic/defective** or saprophytic, but nevertheless have been recovered from nodules of actinorhizal plants and grow well *in vitro* but cannot fulfill Koch's postulates (Hameed et al., 1994). The significance of the latter strains to the symbiosis is unknown, for instance strain CN3 obtained from *Coriaria nepalensis* nodules but unable to nodulate any plant (Mirza et al., 1992) or EuI1c obtained from *Elaeagnus umbellata* that can only induce nonefficient nodules on its host (Baker et al., 1980; Tisa et al., 1983).

Part of the rationale for undertaking the different *Frankia* genome sequencing projects was to identify the bacterial symbiotic determinants and determine evolutionary context for host range and plant–microbe interactions. This concerned a range of plants differing in the symbiotic relationship.

24.2 GENOMES OBTAINED AND UNDERWAY

The first publication of *Frankia* genomes centered on three strains differing in their symbiotic relationships, two were **facultative** (*Frankia* EAN1pec, cluster 3 and *Frankia alni* ACN14a, cluster 1a) and the last one was **facultative/limited** (*Frankia* CcI3, cluster 1b) (Normand et al., 2007).

A fourth genome was published (Persson et al., 2011) from an **obligate** symbiont, strain Dg1 growing in *Datisca glomerata*. Recently, two more genomes were published representing another cluster 1a mid-host range *Frankia*, strain QA3 (Sen et al., 2013) and the first cluster 4 **asymbiotic** atypical *Frankia*, strain CN3 (Ghodbhane-Gtari et al., 2013).

Symbiotic status ranges from obligatively symbiotic (or completely symbiotic, cluster 2) to asymbiotic/defective (saprophytic, cluster 4) with three intermediate categories with varying degrees of autonomy.

Several other genomes are on the way to being published and many should have been published when this chapter appears.

A trend that emerges from Table 24.1 is the link between genome size and symbiotic status although the number of genomes characterized is still small. Smaller genomes appear to have contracted through a combination of gene deletion associated with genome instability (Fig. 24.1). Interestingly, most of the larger genomes have an abundance of duplicated genes, as well as signs of genome instability, that has led to genome expansion (Normand et al, 2007). There is still only a single genome in cluster 2 as well as in cluster 1b that comprises *Casuarina*-infective strains. In cluster 1, the two genomes sequenced are close to one another, but this is not true in cluster 4. The latter may not form a real cluster, since the initial phylogenetic study (Normand et al., 1996) was based on incomplete (<1000 nt) sequences comprising probably numerous sequencing errors with long branches.

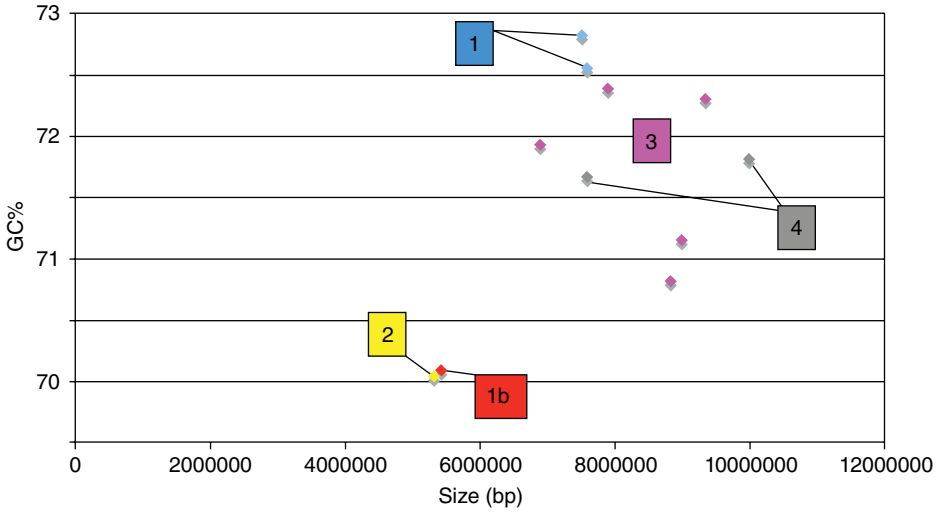

Figure 24.1 Genome sizes (*x*-axis) versus mol% G + C. Genomes from obligately symbiotic (cluster 2) or partially autonomous (cluster 1b) strains are the smallest (5.3–5.5 Mb) and have the lowest G + C% (70%). At the other extreme of the range are the saprophytic strains (asymbiotic/defective, cluster 4) with the largest genomes (up to 9.9 Mb). The other two groups, mostly symbiotic (cluster 1, *Alnus*-infective) and facultatively symbiotic (cluster 3), have intermediate sizes. Cluster 1 strains have the highest G + C% (>72.5%).

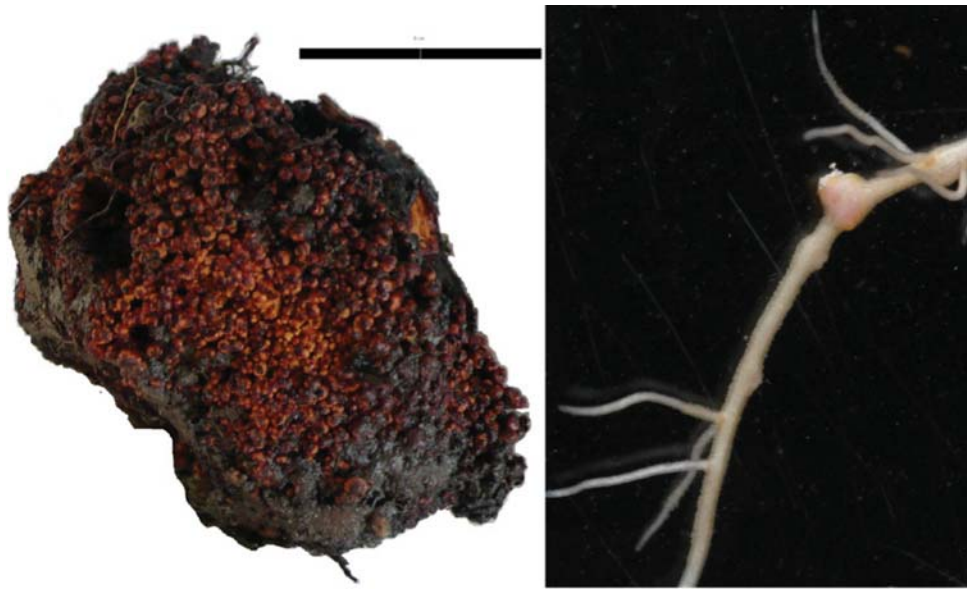

Figure 24.2 *Alnus glutinosa* nodules. Old perennial nodule on a black alder root from a field-grown individual (a; bar = 5 cm). A young *A. glutinosa* root (b) grown *in vitro* and inoculated with *Frankia alni* strain ACN14a that deforms root hairs, penetrates, and induces prenodular swellings that develop into small nodules (reddish swellings, upper right).

24.3 LINK WITH HOST RANGE

24.3.1 Host Biogeography

Cluster 2 (obligate) strains have a scattered distribution, since they appear to be absent from soils in the absence of their host plants, as shown by most trapping studies (personal observations). The host plants are similarly disjunct: *Datisca* spp. are present in California and Pakistan; *Coriaria* in Southern Europe, Eastern Asia, Mexico, North Africa, New Zealand; nodulated Rosaceous *Dryas* in North America and Northern Asia, and the other Rosaceous genera *Purshia*, *Chamaebatia*, and *Cercocarpus* in North America. Furthermore, it is difficult to be certain about the infectivity of these uncultivated symbionts, and it is only their genetic proximity and a few cross-compatibility tests that make us think these strains are relatively nondiverse.

For the most part, cluster 1b strains are uncultivated as isolation attempts from most *Casuarina* spp. and *Allocasuarina* spp. nodules have been unsuccessful. Isolates closely related to CcI3 have been obtained mainly from *C. equisetifolia* and related species, most of which have been deliberately planted outside their region of origin, Australia (Simonet et al., 1999). The isolates as well as the unisolated endophytes are phylogenetically close to one

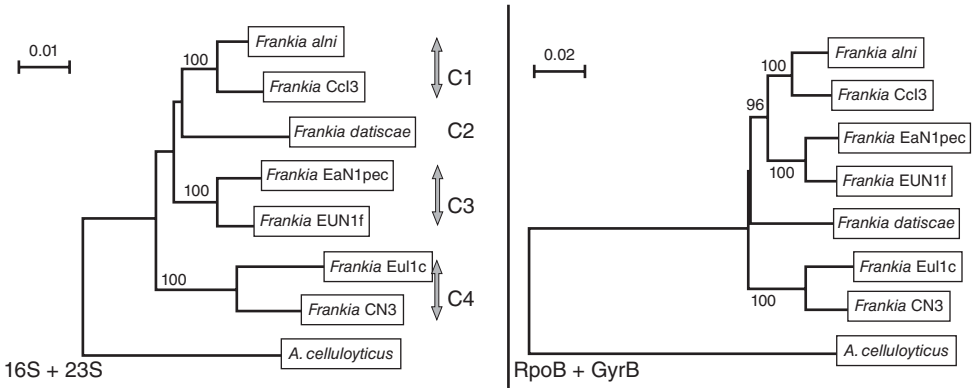

Figure 24.3 Different genes yield different topologies. In the case of *Frankia*, using *Acidothermus cellulolyticus* as outgroup, a combination of 16S + 23S rRNA puts the cluster 4 as the first to emerge, followed by cluster 3, cluster 2, and cluster 1. Conversely, a combination of RpoB + GyrB puts the cluster 4 as the first to emerge, followed by cluster 2, cluster 3, and cluster 1. A more robust multigene phylogeny should help discern the evolutionary history of *Frankia*.

another and are absent from soils where their host plants are not growing (Zimpfer et al., 1999). Presently, *Casuarina* trees grow in most tropical regions of the world and are planted extensively along seacoasts to stabilize the coastal dunes. In these situations, "casuarinas-infective strain isolates" can be found in soils only in close proximity to the trees (Zimpfer et al., 1999).

Cluster 1a strains, such as *Frankia alni* ACN14a, are infective on *Alnus* spp. as well as on *Myrica gale* and *Morella* sp. Alders are circumpolar plants that have adapted to specific niches. Black alder (*A. glutinosa*) may be the best-known actinorhizal plant; it grows along river banks where it sometimes produces large perennial structures (Fig. 24.2). Other *Alnus* species grow in nitrogen-poor biotopes in temperate locations around the globe.

Cluster 3 strains infect plants from the Elaeagnaceae that are also present in temperate ecosystems around the world, predominantly in Asia, Europe, North America, and North Africa. They also infect certain plants from the Rhamnaceae in South America, the Myricaceae, and *Gymnostoma* in Oceania. Finally, they are found as "atypical" strains on *Casuarina* and *Alnus* (Benson and Silvester, 1993).

Myrica gale or *Morella* species that can be nodulated by cluster 1 and 3 strains have a worldwide distribution (Benson and Silvester, 1993).

24.3.2 Core Genome

Several versions of the core *Frankia* genome can be computed: one that would contain genes present in all *Frankia* strains, and the other that is more specific would contain all genes present in strains that are partly or completely symbiotic, presumably including the symbiotic determinants. As the number of sequenced genomes increases over the years, the number of potentially symbiotic genes should approach an asymptote.

At a threshold level of 70% of similarity in amino acid sequences, the four published genomes share a core of around 860 CDS that represent 12.5% of the *F. alni* genome. This number increases to 1765 CDS when the threshold is lowered to 50% (25.7% of genome).

When a set of actinobacteria comprising phylogenetic neighbors *Acidothermus* and *Geodermatophilus* is used, and all shared genes are subtracted from this set, one obtains the frankial specific genome. At a threshold of 70%, that set is composed of 594 CDS or 8.65% of the genome; at a level of 50%, there are 783 CDS or 11.4% of the genome; and at a threshold of 30%, there are 650 CDS or 9.46%, including the *nif* genes.

Having numerous genomes allows one to infer phylogenies beyond what is possible with the 16S rRNA gene that is often used singly for that purpose (Fig. 24.3).

24.4 CONCLUSION

The emerging dataset represented by the *Frankia* genomes has allowed the identification of some tendencies. The first conclusion is that there is a marked variation in genome size that ranges from 5.3 Mb for the cluster 2 genome to 9.98 Mb for a cluster 4 genome. The second conclusion is that size variation is correlated with the closeness of the symbiotic relation and with the G + C% content (Normand et al., 2007).

Evolution of the symbiosis has resulted in a range of phenotypes from fully effective to asymbiotic/defective (atypical), from nodulating to saprophytic, and from obligate to facultative. There seems to be a geographical component to at least some of the phenotypes. Thus, geographically isolated plants, such as *Casuarina* sp. evolved in Australia and some Pacific islands, with fossil evidence for the Casuarinaceae in South America and New Zealand. *Casuarina* symbionts, which have a narrow host range,

appear limited to soils beneath their hosts as casuarinas planted outside their native range generally do not nodulate (Zimpfer et al., 1999). In the case of the obligate symbionts found in nodules from the North American *Ceanothus* sp., the Rosaceae, Datiscaceae, and Coriariaceae, the plants have developed as disjunct populations, in relative isolation from related plants, leading to the hypothesis that geographical isolation leads to an increasing dependence on symbiosis (Simonet et al., 1999). Plants that are widely distributed (*Alnus*, members of the Elaeagnaceae and Myricaceae) are infected by *Frankia* lineages that retain a wide distribution, even in the absence of cognate hosts (Benson et al., 2004).

The coming increase in sequenced genomes from strains with new phenotypes should help buttress and refine these findings.

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Chapter 25

Core and Accessory Genomes of the Diazotroph *Azospirillum*

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25.1 INTRODUCTION

Bacteria of the genus *Azospirillum* (Alphaproteobacteria) have been known for many years as plant growth-promoting rhizobacteria (PGPR; see Chapter 90). These free-living nitrogen-fixing and highly motile bacteria can be isolated from the rhizosphere of many grasses and cereals, under tropical and temperate climates (Döbereiner et al., 1976; Patriquin et al., 1983; see Chapter 88). Azospirilla are predominantly root surface-colonizing bacteria, but some strains can also colonize the interior of roots (Elbeltagy et al., 2001; Rothballer et al., 2003). Through the establishment of cooperation (associative symbiosis) with plants, Azospirilla exert beneficial effects on plant growth and yield of many agronomically important crops such as rice, maize, and wheat while benefiting in return from root exudates (Okon, 1985; Veresoglou and Menexes, 2010; see Chapter 90). This plant's stimulatory effect has been attributed to several mechanisms, the two main properties that define the genus being the most documented: production of plant growth-promoting substances (such as auxins) and biological nitrogen (N) fixation. Apart from these two mechanisms, other properties are thought to be implicated in plant growth promotion, and it is likely that the *Azospirillum* effect results from a combination of different mechanisms (Bashan and de-Bashan, 2010). Moreover, *Azospirillum* spp. can, to a lesser extent, enhance plant health by inhibiting plant parasites (Miché et al., 2000) and/or by stimulating the plant defense (Yasuda et al., 2009).

In order to rationalize the use of *Azospirillum*, genetic studies have mainly focused on plant beneficial traits (nitrogen fixation, auxin biosynthesis) and properties linked to root colonization and survival in the rhizosphere (Bashan et al., 2004; Steenhoudt and Vanderleyden, 2000; Fibach-Paldi et al., 2012). Despite these studies, the growth response of plants to *Azospirillum* inoculation is not completely predictable and differential varietal response has been reported for several crops, including wheat, sorghum, corn, and rice (Drogue et al., 2012; García de Salamone et al., 2010; Sasaki et al., 2010; Saubidet and Barneix, 1998; Vargas et al., 2012;

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Veresoglou and Menexes, 2010; see Chapter 90). To provide new insights into the *Azospirillum*–plant cooperation, genomes of several *Azospirillum* strains belonging to different species were recently sequenced and published. This review aims at summarizing the most important features revealed by comparative genome analyses.

25.1.1 Size and Architecture of *Azospirillum* Genomes

Pioneer studies have revealed that *Azospirillum* genomes are consisting of multiple replicons, and their sizes vary among species from 4.8 to 9.7 Mpb (Caballero-Mellado et al., 1999; Martin-Didonet et al., 2000). Until now, the genomes of five strains belonging to different species, isolated from various host plants and locations, were sequenced and published: (i) *Azospirillum* sp. B510, a strain isolated from disinfected rice stems in Japan and closely related to the species *Azospirillum oryzae* and *Azospirillum zeae* (Elbeltagy et al., 2001; Kaneko et al., 2010; Wisniewski-Dyé et al., 2012); (ii) *Azospirillum amazonense* Y2 isolated from the gramineous plant *Hyparrhenia rufa* in Brazil (Magalhães et al., 1983; Sant'Anna et al., 2011)); (iii) *Azospirillum brasilense* Sp245 isolated from wheat in Brazil (Baldani et al., 1986; Wisniewski-Dyé et al., 2011); (iv) *A. brasilense* CBG497 isolated from maize grown on an alkaline soil (pH 8) in the northeast of Mexico and used as a commercial biofertilizer (Garcia-Olivares et al., 2007; Wisniewski-Dyé et al., 2012); and (v) *Azospirillum lipoferum* 4B isolated from rice in France (Thomas-Bauzon et al., 1982; Wisniewski-Dyé et al., 2011). Genome size ranges from 6.5 Mbp (*A. brasilense* CBG497) to 7.6 Mbp (*Azospirillum* sp. B510) (Table 25.1). All sequenced genomes are composed of multiple circular replicons, whose number varies from four (*A. amazonense* Y2) to seven (Table 25.1).

Separation of plasmids by the Eckhardt method and pulsed-field gel electrophoresis (PFGE) analyses performed on other unsequenced strains repeatedly demonstrate the presence of multiple replicons (our unpublished data), implying that a composite genome architecture is a rule for members of the *Azospirillum* genus. Thus, *Azospirillum* genome architecture is more similar to the one observed in rhizobia (Rhizobiaceae family) (Crossman et al., 2008; González et al., 2006) than to the one observed in its closest relatives. Indeed, genomes of others, Rhodospirillaceae (such as *Rhodospirillum*, *Magnetospirillum*), display sizes from 4.3 to 5 Mpb composed of a unique chromosome or a chromosome and a tiny plasmid (<50 kb).

For all strains (except *A. amazonense* Y2 for which the information is not available), the analyses of GC skew shift point (Lobry, 1996) and stress-induced destabilization site (SIDD) (http://www.genomecenter.ucdavis.edu/benham/sidd/websidd.php) allowed to locate the putative chromosomal origin (*Cori*) of replication on the largest replicon (ca. 3 Mbp). The *Cori* region contains three to four DnaA boxes, and it is found adjacent to the *hemA* locus that is similar to the *Cori* pattern of many other Alphaproteobacteria (Brassinga et al., 2001). All the other replicons have *repABC/parAB* plasmid-type replication systems; with a clear SIDD site and the presence of several short (direct, inverted, tandem) repeats and palindromes, as well as a gene encoding a protein highly similar to the replication initiator protein. Some of the latter have been classified as chromids (up to five in the genomes of *A. lipoferum* 4B and *Azospirillum* sp. B510), that is, they display plasmid-type maintenance replication systems, presence of essential genes, and a nucleotide composition close to that of the chromosome (Harrison et al., 2010; Acosta-Cruz et al., 2012; Wisniewski-Dyé et al., 2011; Wisniewski-Dyé et al., 2012). In finished and closed genomes, a total of eight or nine ribosomal operons have been detected on the chromosome and on various chromids (Table 25.1); interestingly, the p1 chromid always bears the highest number of ribosomal operons (up to four in *Azospirillum* sp. B510). The smallest replicon, p6, is a typical plasmid and exhibits the lowest average GC content, which suggests an external origin by horizontal gene transfer (HGT); such an observation was previously made for two other Alphaproteobacteria interacting with plants, *Rhizobium etli* and *Rhizobium leguminosarum* (Crossman et al., 2008). Chromids and plasmids comprise the largest proportion of the total genome, with 55.2% for *A. brasilense* CBG497, 56.4% for *A. lipoferum* 4B and *Azospirillum* sp. B510, and 59.8% for *A. brasilense* Sp245. Therefore, in addition to possessing the largest number of chromids among all prokaryotic genomes sequenced to date, *Azospirillum* has the biggest proportion of its genome on nonchromosomal replicons (Wisniewski-Dyé et al., 2012).

25.1.2 Genome Plasticity and Horizontal Gene Transfer

A comparison of three *Azospirillum* genomes (those of *Azospirillum* sp. B510, *A. lipoferum* 4B, and *A. brasilense* Sp245) revealed very little synteny between replicons (Wisniewski-Dyé et al., 2011). Indeed, alignment of *Azospirillum* sp. B510 and *A. lipoferum* 4B genomes displayed little synteny on the chromosome and none on the other replicons despite an average nucleotide identity (ANI) of 91% and a relative distance of 0.01 between the two genomes (Fig. 25.1). Alignment of *A. lipoferum* 4B and *A. brasilense* Sp245 genomes revealed no synteny despite an ANI value of 89% and a relative distance of 0.10 (Fig. 25.1).

When such a comparison was made on two rhizobia of comparable genetic relatedness (*Rhizobium etli* CFN42/*R. leguminosarum* biovar *viciae* 3841, ANI = 89%), a strong synteny was observed on nearly all replicons (Wisniewski-Dyé et al., 2011). This indicates that *Azospirillum* genomes have undergone numerous rearrangements

Table 25.1 Genomic features of *Azospirillum* genomes sequenced to date

Strain and Features	Chromosome	p1	p2	p3	p4	p5	p6	Total (pb)
***A. lipoferum* 4B**								
Size of replicon*, †	2,988,332*	**1,040,425***	**750,123***	**648,491***	**645,253‡**	**478,032***	295,744	6,846,400
G + C content	67.6	67.6	67.6	67.8	68.3	67.7	67.1	67.7
Number of ORFs	2,904	883	640	555	599	415	237	6,233
***Azospirillum* sp. B510**								
Size of replicon	3,311,395*	**1,455,109***	**723,779***	**681,723***	**628,837**	**537,299***	261,596	7,599,738
G + C content	67.8	67.6	67.5	67.4	68.0	67.5	65.9	67.6
Number of ORFs§	3,287	1,263	693	589	598	464	232	7,126
***A. brasilense* Sp245**								
Size of replicon	3,023,440*	**1,766,028***	**912,449***	778,798	**690,334***	191,828	167,364	7,530,241
G + C content	68.6	68.6	68.3	68.2	69.0	66.7	66.8	68.5
Number of ORFs	3,309	1,812	922	824	691	163	125	7,846
***A. brasilense* CBG497**								
Size of replicon	2,900,071	**1,598,241***	**731,389***	488,405**	**606,415‡**	Absent	148,687	6,473,208
G + C content	68.4	68.8	68.8	66.05	69.3		67.1	68.4
Number of ORFs	2,895	1,430	643	512	583		122	6,185
***A. amazonense* Y2††**								
Size of replicon	~2,700,000*	~2,200,000	~1,700,000*	~750,000	Absent	Absent	Absent	~7,350,000
G + C content	nk	nk	nk	nk				nk
Number of ORFs	nk	nk	nk	nk				3,319

nk, not known.

*Those replicons carry ribosomal operons (sometimes partial). This information is partial for *A. brasilense* CBG497.

†When the size is indicated in bold, the chromid definition applies to the corresponding replicon, that is, plasmid-type maintenance replication systems, presence of essential genes, and a nucleotide composition close to that of the chromosome (Harrison et al., 2010; Wisniewski-Dyé et al., 2011).

‡The third criteria of chromid definition (nucleotide composition close to that of the chromosome) does not apply for these two replicons.

§The number of ORFs corresponds to the one established after the sequence was imported and annotated into the MaGe platform (Vallenet et al., 2009).

**According to PFGE data, the sequence of size of this replicon is 650 kb versus a sequenced size of 488 kb; consequently, genome coverage is estimated at 97–98% (Wisniewski-Dyé et al., 2012).

††Sizes of replicons are indicative as no information on genome architecture was provided when the genome was sequenced (Sant'Anna et al., 2011); hence, data on the sizes of replicons and on the presence of ribosomal operons are derived from PFGE analyses and hybridization with a 16 s rDNA probe performed anteriorly (Martin-Didonet et al., 2000). The low number of ORFs is far to what is expected for a 7.3 Mbp genome size and might be attributable to the draft status of this genome (1617 contigs) (Sant'Anna et al., 2011). The eventual chromid status of the replicons could not be assigned for the same reason.

compared to rhizobial genomes usually recognized for their plasticity. Experimental evidence had already highlighted the extraordinary genome plasticity of *Azospirillum*. For instance, the appearance of phenotypic variants was correlated with plasmid loss or reorganization (Vial et al., 2006), while plasmid content of strain *A. brasilense* Sp245 collected from several laboratories showed discrepancies (Pothier et al., 2008). Moreover, the presence of bacteriophages was evidenced, with some strains hosting gene-transfer agents that typically package bacterial genome fragments (Boyer et al., 2008). In addition, a thorough analysis of *Azospirillum* sp. B510 and *A. lipoferum* 4B genomes disclosed the presence of many (direct and palindromic) repetitive sequences of a size superior to 80 pb as well as clustered regularly interspaced short palindromic repeats (CRISPR) sequences that could act as recombination hotspots; insertion sequences (IS) are also scattered throughout all replicons and represent up to 4.47% of the genome in *Azospirillum* sp. B510 (310 IS, including 176 full IS) (Wisniewski-Dyé et al., 2011). However, despite experimental evidence of genome plasticity and presence of relevant elements in the genomes, the detailed mechanisms underlying genomic rearrangements remain to be elucidated.

Interestingly, although being multireplicon Alphaproteobacteria interacting with plants (such as rhizobia), the genus *Azospirillum* belongs to the family Rhodospirillaceae whose nearly all other known representatives live in aquatic habitats. In order to determine which genes *Azospirillum* shares with its aquatic relatives and what was the origin of its additional genes, a robust scheme for detecting ancestral genes and those acquired by HGT genes was developed (Wisniewski-Dyé et al., 2011). Briefly, proteins were assigned as being ancestral or horizontally transferred, with varying degrees of confidence, based on the

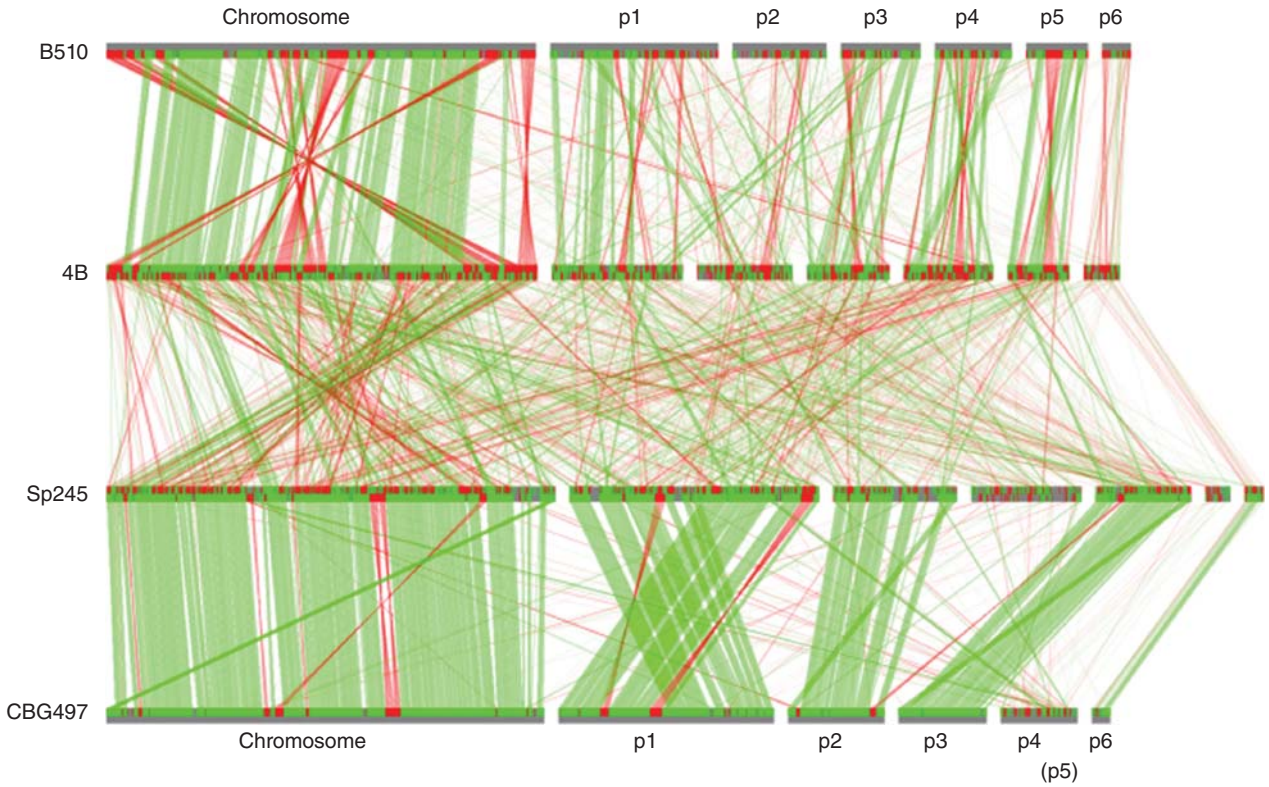

Figure 25.1 Whole genome alignments of *Azospirillum* replicons. Each line represents one gene; a green line indicates that the two orthologs are in the same orientation, whereas a red line indicates that the two orthologs are in an opposite orientation.

presence of members of Rhodospirillales and Rhodospirillaceae in the top eight BLAST hits. With this scheme, most protein-coding genes of *Azospirillum* could be classified as ancestral or HGT and, remarkably, nearly half of the genes whose origins could be resolved appear to be horizontally transferred (Wisniewski-Dyé et al., 2011). When the genomes of other Rhodospirillaceae were subjected to the same analysis, a substantially lower HGT level was revealed, whereas the number of ancestral genes in all organisms was comparable. The ancestral set of genes principally contained genes involved in "housekeeping" functions such as translation, posttranslational modification, cell motility, and nucleotide and coenzyme transport and metabolism. The HGT set contained a large proportion of genes involved in specific dispensable functions, such as defense mechanisms, cell wall biogenesis, transport and metabolism of amino acids, carbohydrates, inorganic ions, and secondary metabolites. A great proportion of HGT genes exhibit similarity with genes of terrestrial and plant-associated bacteria, notably Rhizobiales and Burkholderiales. Thus, HGT, likely promoted by conjugation or transduction events, may have been a major driving force in the transition of *Azospirillum* from aquatic to terrestrial environments, as well as in adaptation to the plant rhizosphere, an environment rich in amino acids, carbohydrates, inorganic ions, and secondary metabolites. Moreover, separation of *Azospirillum* from their close aquatic relatives coincided approximately with the emergence of vascular plants on land, about 400 million years ago (Wisniewski-Dyé et al., 2011).

25.1.3 The *Azospirillum* Core Genome

A reciprocal best blast hit criterion has been used previously to identify the most probable set of orthologous proteins shared by four fully sequenced *Azospirillum* strains. A total of 2328 proteins (named the AZO-core) appeared to be shared by the four strains, representing between 30% and 38% of the total encoded proteins within a genome (Wisniewski-Dyé et al., 2012). The repartition among replicons showed that the AZO-core was mainly chromosomally encoded (from 62% to 65% according to the strain considered), whereas the nonchromosomal proportion of AZO-core was unevenly distributed among strains. Not surprisingly, the AZO-core set revealed to be dominated by proteins of ancestral origin (74%) but contains more than a fifth (22%) of proteins encoded by horizontally acquired genes and a small proportion of proteins encoded by genes whose origin could not be resolved (4%; see also Chapters 26, 27).

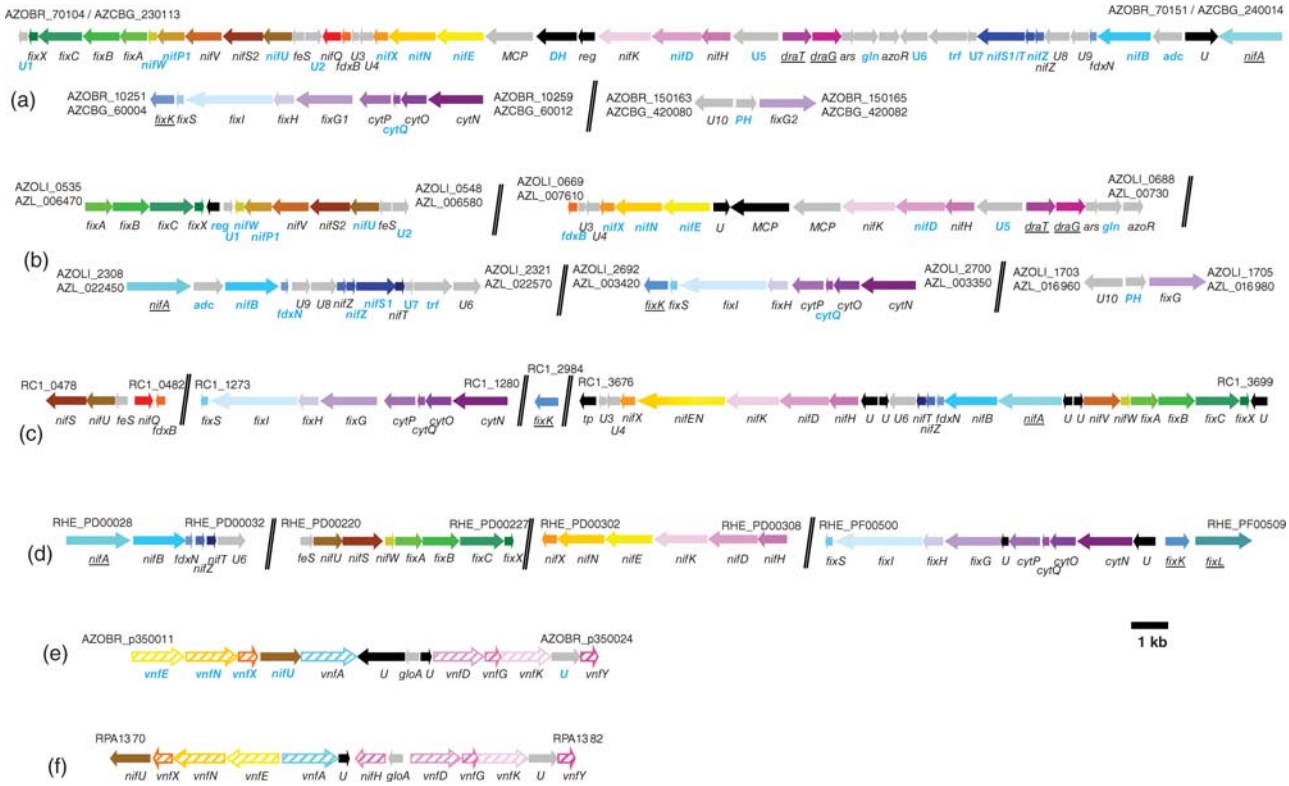

Figure 25.2 Organization of *Azospirillum* nitrogen fixation genes. Genes encoding FeMo nitrogenase of *Azospirillum brasilense* Sp245 and CBG497 (a); *A. lipoferum* 4B and *Azospirillum* sp. B510 (b); *Rhodospirillum centenum* SW (c); *Rhizobium etli* CFN42 (d). Genes encoding vanadium nitrogenase of *Azospirillum brasilense* Sp245 (e) and *Rhodopseudomonas palustris* CGA009 (f). Genes involved in nitrogen fixation are represented by colored arrows. Black and gray arrows represent genes unrelated to nitrogen fixation: black arrows represent genes that are unique to one strain whereas gray arrows represent conserved genes. For black and gray arrows, the meaning of gene names is as follows: *adc* (putative adenylate/guanylate cyclase), *ars* (putative arsenate reductase), *azoR* (NADH-azoreductase), *DH* (dehydrogenase), *feS* (Fe-S cluster assembly protein), *gln* (putative glutamine amidotransferase), *gloA* (lactoylglutathione lyase), *MCP* (putative methyl-accepting chemotaxis receptor), *PH* (phosphohydrolase), *reg* (putative regulator), *tp* (transposase), *trf* (putative aminotransferase), and *U* (protein of unknown function). A gene name depicted in black indicates an ancestral gene, whereas a gene name appearing in blue indicates a gene that has been horizontally acquired (Wisniewski-Dyé et al., 2011). Gene names that are underlined encode regulators of nitrogen fixation; *rpoN* (encoding sigma 54) is not depicted in this figure. Sp245 possesses two chromosomally encoded copies of *nifQ* (AZOBR_70116 represented here, and AZOBR_90008); homologs of AZOBR_90008 can be found in *A. lipoferum* (AZOLI_0980), *R. centenum* SW (RC1_0481), and *R. etli* CFN42 (RHE_PD00259) and are not represented here. All strains contain at least one copy of *nifP* (not always depicted here); *Azospirillum* strains contain two copies of *nifP* (only one is represented here). *R. etli* CFN42 lacks a *nifV* homolog. *draT* and *draG* are absent from the genomes of *R. centenum* SW and that of Rhizobiales.

The majority of genes for nitrogen fixation, one of the main features that define the *Azospirillum* genus, belong to the AZO-core. However, discrepancies in gene organization are found from one strain to another. In the two *A. brasilense* strains (Sp245 and CBG497), nitrogen-fixation genes lie on two chromosomal clusters (a big cluster of ca. 41.4 kb, and a second cluster of ca. 9.3 kb), whereas in *A. lipoferum* 4B and *Azospirillum* sp. B510, five chromosomal clusters have been identified (Fig. 25.2). Genes encoding the main structural components as well as regulators of nitrogen fixation genes (such as *fixK*, *nifA*, *draT*, *draG,* and *rpoN*) fall into the ancestral set (Fig. 25.2).

Rhodospirillum centenum SW lacks *draT* and *draG* allowing the posttranslational regulation of dinitrogenase reductase by adenosine diphosphate (ADP)-ribosylation (see Chapters 12, 13), but these genes are present in other *Rhodospirillum* strains and in *Magnetospirillum*. This regulation mechanism is also absent from the genomes of Rhizobiales but additional regulators, that are absent in *Azospirillum* (such as FixL and FixJ), have been reported. Interestingly, several nitrogen-fixation genes, although present in some aquatic relatives, fall into the HGT set due to strong homology with genes of other nitrogen fixers (Fig. 25.2). The unique structural gene falling into this category (i.e., *nifD* encoding the alpha chain of FeMo nitrogenase) may have been acquired from Rhizobiales, whereas all others are related to accessory functions (such as nitrogenase carrier protein NifX and FeMo-cofactor scaffold and assembly proteins NifN and NifB). Thus, although

encoding an ancestral function, nitrogen-fixation genes may have several origins in *Azospirillum*.

Among the 2328 proteins of the AZO-core, half (1151) of them was shown to be shared by *Azospirillum* strains and by the phylogenetically related *R. centenum* SW, and encode mainly "housekeeping" functions. Genes belonging to the AZO-core that are absent in *R. centenum* SW have been mainly acquired horizontally (53%) or are shared with other aquatic relatives (40%) or their origin could not be resolved (7%). Those genes are principally involved in signal transduction, regulation of transcription, carbohydrate transport and metabolism, and amino acid transport and metabolism; this latter category also includes organic acids, compounds that are abundant in the rhizosphere. Transporters, especially ATP binding cassette (ABC) transporters, are overrepresented. They can serve to internalize the wide diversity of organic and mineral compounds present in the rhizosphere, such as organic compounds exuded by plant roots, or to expel putative plant toxic compounds via multidrug resistance (MDR) efflux pumps. Thus, compared to its closest aquatic relative *R. centenum*, *Azospirillum* is more adapted to life in fluctuating environments such as the rhizosphere.

As for genes involved in direct plant growth promotion, only the pyrroloquinoline quinone (PQQ) operon, allowing the synthesis of the cofactor PQQ, a compound displaying plant growth-promoting properties belongs to the AZO-core (Choi et al., 2008); other genes belong to the accessory genome and may have been acquired specifically after speciation events or by individual strains (see later).

25.1.4 Accessory Genome and Its Relevance for Interaction with Plants and for Host Specificity

Chemotaxis toward plant-exuded compounds and motility are primordial for the initiation of root colonization in a wide range of rhizobacteria. The AZO-core contains genes implicated in flagellum biosynthesis and four common chemotaxis operons, whereas one and two additional chemotaxis operons are carried by the genomes of *A. lipoferum* 4B and *Azospirillum* sp. B510, respectively (Wisniewski-Dyé et al., 2011). Consequently, those two strains contain a significant number of unique genes encoding methyl-accepting proteins, able to detect various physicochemical cues and to relay information to the flagellar motors via a signal transduction cascade. Once colonization is achieved, polysaccharides and other adhesion structures mediate bacterial attachment to plant roots. Next to a common set of genes involved in the biosynthesis of exopolysaccharide (EPS) and lipopolysaccharide (LPS) such as *noeL*, *noeJ,* and *rmlD* (Jofré et al., 2004; Lerner et al., 2009), the presence of several unique genes suggests that EPS and LPS components might differ from one *Azospirillum* strain to another, a feature previously reported for LPS (Jofré et al., 2004). Cellulose synthesis allows bacteria to bind tightly to the roots (Rodriguez-Navarro et al., 2007), and genes involved in cellulose synthesis and acquired by HGT (Fig. 25.3) are found exclusively in *A. lipoferum* 4B and *Azospirillum* sp. B510.

TAD (Tight ADhesion) pili, present exclusively in the *A. brasilense* species, play an essential role in biofilm formation, colonization, and pathogenesis in various genera (Tomich et al., 2007), and their role in biofilm formation on abiotic surfaces was recently assessed in *A. brasilense* Sp245 (Wisniewski-Dyé et al., 2011). Cellulases and hemicellulases likely contribute to endophytic plant colonization, a property described for *A. brasilense* Sp245 and *Azospirillum* sp. B510 (Assmus et al., 1995; Elbeltagy et al., 2001). A weak cellulolytic activity was detected for those two strains and for *A. lipoferum* 4B (not known as an endophyte), and a substantial number of glycosyl hydrolases (from 26 to 34) were identified in *Azospirillum* genomes, with some CAZy families being restricted to a species (Wisniewski-Dyé et al., 2011). Altogether, these features might be relevant for the colonization of specific hosts.

While various studies showed evidence that the plant growth-promoting effects of *Azospirillum* depend on both host plant genotypes and bacterial strains (Sasaki et al., 2010; Saubidet and Barneix, 1998; Vargas et al., 2012; Veresoglou and Menexes, 2010), the molecular bases of these host-specific responses are still not elucidated. It was recently suggested that host specificity of phytostimulating cooperations could be the result of successive genotype-dependent responses in the three-step establishment of such symbioses: (i) attraction of bacteria from the surrounding soil to the rhizosphere; (ii) attachment to the root surface; and finally, (iii) functioning of cooperation that becomes effective (Drogue et al., 2012). In the case of *Azospirillum*, this effective functioning is thought to occur through nitrogen fixation and modulation of the plant hormonal balance (Bashan and de Bashan, 2010; Wisniewski-Dyé et al., 2013), two properties that are differentially distributed among *Azospirillum* genomes.

Indeed, next to nitrogen-fixation genes that have been inherited vertically and that are chromosomally encoded, *A. brasilense* Sp245 possesses a second cluster for nitrogen fixation, encoding a vanadium nitrogenase, and located on a chromid (Fig. 25.2e). This cluster, bordered by a gene encoding an integrase, seems to have been horizontally acquired from *Rhodopseudomonas palustris*; synteny analysis between the two operons indicates that this region has undergone rearrangements (Fig. 25.2). Its absence from the genome of *A. brasilense* CBG497 suggests that its acquisition by HGT is rather recent. Whether this vanadium nitrogenase is expressed and what are its inducing conditions remain to be determined as a proteomic analysis of *A. brasilense* Sp245 cells grown under nitrogen-fixation

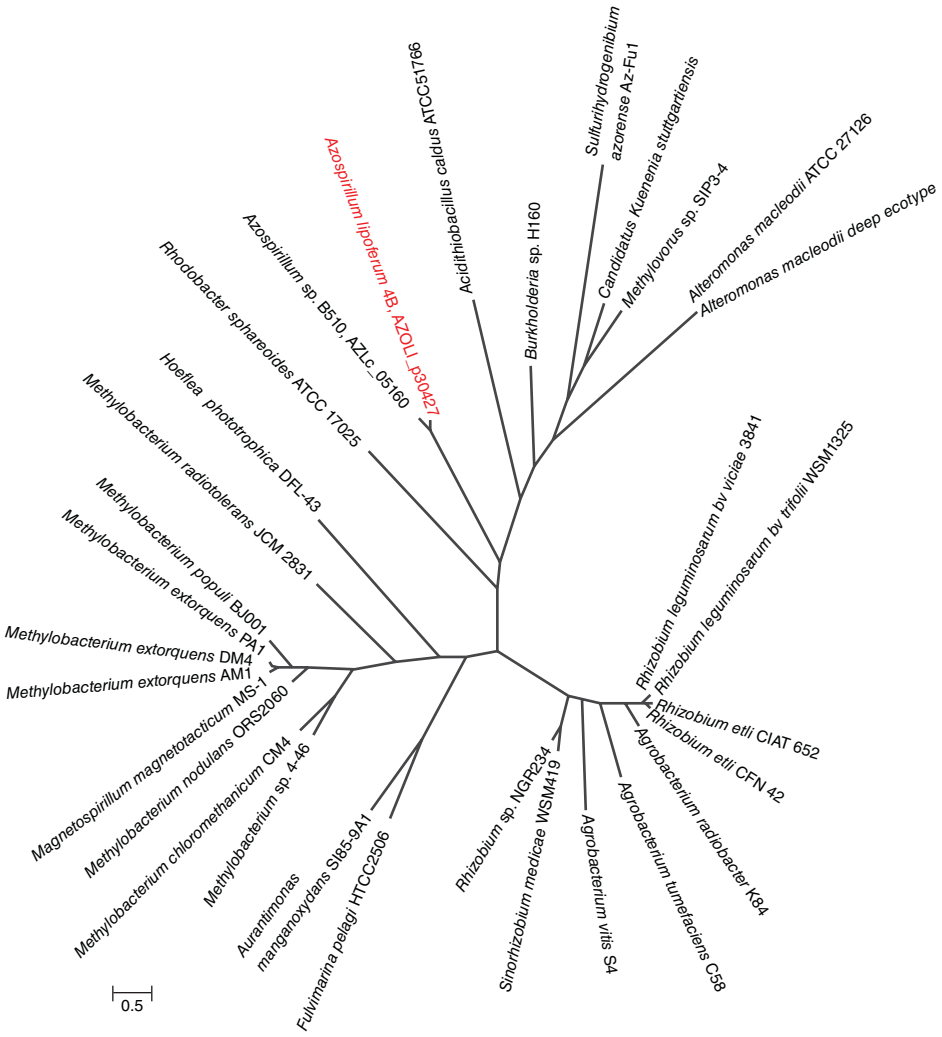

Figure 25.3 Phylogenetic tree for cellulose synthase of *Azospirillum lipoferum* 4B. Tree was built from aligned sequence of the *A. lipoferum* 4B query (*celB*, AZOLI_p30427) and 30 most similar sequences were determined by BLAST.

conditions did not allow to detect this low-abundant protein (Wisniewski-Dyé et al., 2011).

Interestingly, *A. lipoferum* 4B and *Azospirillum* sp. B510 may have acquired the ability to secrete gluconic acid and hence to solubilize inorganic phosphates, another important trait for enhancement of plant nutrition and scarcely reported for the *Azospirillum* genus (Rodriguez et al., 2004).

Apart from plant nutrition improvements, *Azospirillum* can modulate the plant hormonal balance via the synthesis or via the degradation of phytohormones or precursors of phytohormones (Bashan et al., 2010; Wisniewski-Dyé et al., 2013).

A. brasilense Sp245 is well known to produce indole-3-acetic acid (IAA) from tryptophan (Trp) through the indole-3-pyruvate (IPyA) biosynthetic pathway (Spaepen et al., 2007; see Chapter 91). One key gene of this pathway, *ppdC*/*ipdC*, encoding IPyA decarboxylase, is present in the sequenced genomes of the two *A. brasilense* strains but absent from those of *A. lipoferum* 4B and *Azospirillum* sp. B510 (Fig. 25.4).

The first and third steps of the IPyA pathway are catalyzed by enzymes, respectively, aromatic aminotransferases and NAD-dependent aldehyde dehydrogenase, which are common and nonspecific enzymes. Recently, the contribution of *hisC1*, which encodes an aromatic amino acid aminotransferase-1 (AAT1), to IAA biosynthesis was shown to occur in *A. brasilense* Sp7 (Castro-Guerrero et al., 2011); homologs sharing identity levels higher than 75% with AAT1 from Sp7 were found in all four *Azospirillum* genomes. Other metabolic pathways are known to contribute to IAA biosynthesis; an *ipdC* knockout mutant of *A. brasilense* Sp245 still produced 10% of the wild-type IAA level (Spaepen et al., 2007). Genetic determinants putatively involved in other pathways and classified as HGT were highlighted in specific strains (Fig. 25.4) (Wisniewski-Dyé et al., 2011). Functional studies are required to validate the involvement of those genetic determinants in IAA biosynthesis in those strains, as high-performance liquid chromatography (HPLC) analyses revealed the ability to

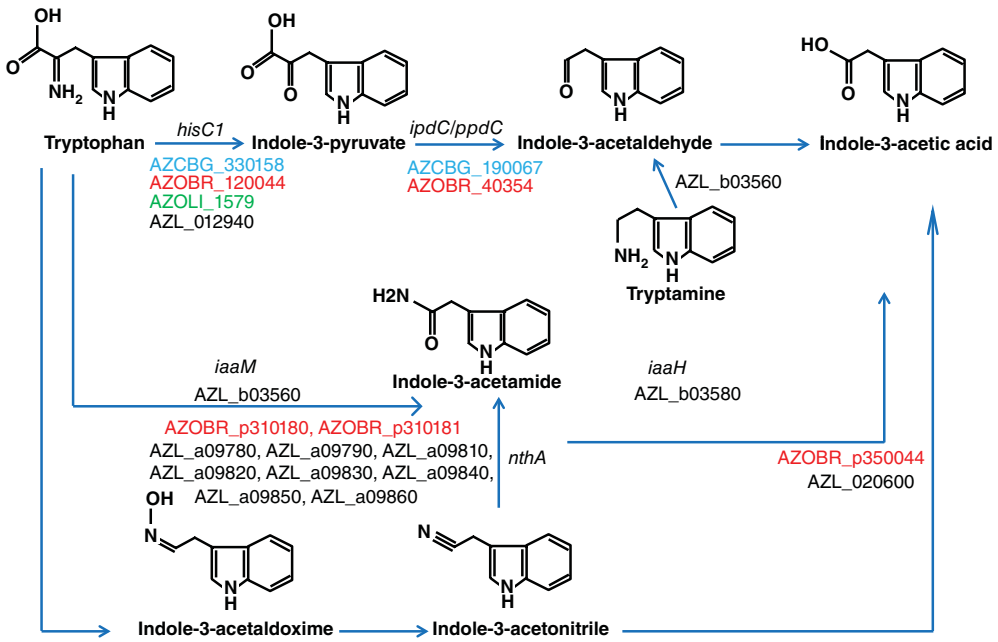

Figure 25.4 *Azospirillum* genes putatively involved in IAA biosynthesis. Genes from *A. brasilense* CBG497 and Sp245 are indicated in blue and red, respectively. Genes from *A. lipoferum* 4B are indicated in green. Genes from *Azospirillum* sp. B510 are indicated in black.

produce IAA in the presence of tryptophan for *A. brasilense* Sp245 and CBG497 but this production was shown to be negligible for *A. lipoferum* 4B and *Azospirillum* sp. B510 (our unpublished data). Interestingly, *A. lipoferum* 4B and *Azospirillum* sp. B510, although not carrying the *ipdC* gene, are able to metabolize IAA; this property previously reported for some rhizobacteria such as *Pseudomonas putida* relies on the presence of the *iac* genes (for IAA catabolism) (Leveau and Gerards, 2008) whose homologs are present only in *A. lipoferum* 4B and *Azospirillum* sp. B510 and have been classified as HGT.

The deamination of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of plant ethylene, is another key activity involved in the modulation of the plant hormonal balance by rhizobacteria. Because ethylene inhibits root growth and may be produced in too large amounts during plant stress response, bacterial ACC deamination can enhance both root system development and plant stress tolerance (Glick et al., 2007). The *acdS* gene, encoding ACC deaminase activity, is harbored by strains of the *A. lipoferum* species and has been acquired by HGT (Prigent-Combaret et al., 2008; Wisniewski-Dyé et al., 2011).

The plant hormonal balance might also be modulated by the degradation of salicylate into catechol via salicylate 1-monooxygenase (EC 1.14.13.1). The corresponding gene has been identified in *A. lipoferum* 4B and in *A. brasilense* genomes and may be the result of two independent acquisitions through HGT (Wisniewski-Dyé et al., 2011). However, only *A. lipoferum* 4B harbors the metabolic pathway for catechol degradation and thus may use salicylate as a source of energy and carbon.

25.1.5 Accessory Genome and Rhizosphere Competence

25.1.5.1 Carbon, Nitrogen, and Phosphate Acquisition. Many genes encoding functions critical for survival in the rhizosphere, such as catabolic properties, are strain specific and are often among those acquired by HGT (Wisniewski-Dyé et al., 2011). A complete ribose degradation pathway was identified in *A. lipoferum* 4B and *Azospirillum* sp. B510 involving a ribokinase (*rsbK*), a deoxyribokinase/ribokinase (*deoK*), and a deoxyribose mutarotase (*deoM*). The catabolic pathway of myoinositol was identified only in *Azospirillum* sp. B510. This strain may use the aliphatic amine methylamine as a nitrogen source; the eight-gene cluster necessary for glutamate-mediated methylamine utilization (composed of *mgdABCD*, *gms*, and *mgsABC*) is present in methylotrophs such as *Methyloversatilis universalis* FAM5 (Latypova et al., 2009) and also in various Rhizobiales from which it may have been acquired. *A. lipoferum* 4B and *Azospirillum* sp. B510 have the ability to degrade the organophosphonate 2-aminoethylphosphonate. Organophosphonates are primarily components of phosphonolipids, and also as constituents of polysaccharides, glycoproteins, glycolipids, and several antibiotics.

The accessory genome also encodes metabolic properties of ancestral origin that have been retained in some

strains. One such example is autotrophy achieved by enzymes of the Calvin–Benson–Bassham (CBB) cycle, whose corresponding operon is present in *A. lipoferum* 4B and in *A. amazonense* Y2. Chemolithoautotrophic growth using CO_2 as a carbon source and H_2 as an energy source was observed for these two strains (our unpublished data). *A. lipoferum* 4B also displays formate-dependent autotrophic growth. Occurrence of the *cbb* operon, investigated using a PCR approach targeting *cbbL* (encoding ribulose-1,5-bisphosphate carboxylase), was shown to occur in 12 out of 40 *Azospirillum* strains isolated from various host plants and geographic locations (our unpublished data). The fact that strains evolving in an environment rich in organic compounds, such as the rhizosphere, have kept the ability to fix CO_2 is puzzling but this property is not restricted to the *Azospirillum* genus. Whether the presence of the *cbb* operon is due to a stochastic inheritance from a phototrophic ancestor or the result of an adaptive evolution to a particular ecological environment is unknown.

25.1.5.2 Degradation of Plant Aromatic Compounds. Aromatic compounds (i.e., organic molecules containing one or more aromatic rings mainly produced by plants) are abundant in plant exudates and may serve as carbon source for microbes. The aerobic catabolism of aromatic compounds usually involves the oxygenolytic hydroxylation of the aromatic ring, producing central dihydroxylated aromatic intermediates (such as catechol and protocatechuate). These intermediates are then cleaved by different types of ring-cleavage dioxygenases, generating aliphatic compounds that funnel into the tricarboxylic acid (TCA) cycle through a small number of central pathways. *A. lipoferum* 4B and *Azospirillum* sp. B510 can degrade catechol and protocatechuate via the β-ketoadipate pathway; they also possess enzymes allowing the conversion of benzoate and 4-hydroxybenzoate into protocatechuate. In addition, *Azospirillum* sp. B510 can convert benzoate into catechol. Both strains can also metabolize gentisate (2,5-dihydroxybenzoate), and adjacent to the cognate cluster, a gene encoding a hydroxybenzoate transporter displaying strong identity with PcaK of *Ralstonia*, a chemoreceptor for aromatic acids (Harwood et al., 1994) is found. Additional hydroxybenzoate transporters are encoded by the genome of *Azospirillum* sp. B510. *A. brasilense* strains seem to be less versatile as only the meta-cleavage pathway of protocatechuate degradation has been identified; catabolism of gallate and methylgallate might be present but one of the key enzymes (EC 4.2.1.83) could not be identified. Experimentally, growth on protocatechuate as the sole carbon source was observed for all strains but *A. brasilense* CBG497 (Wisniewski-Dyé et al., 2012).

The phenylacetate catabolic pathway is the central route where catabolic pathways of many aromatic compounds converge and are directed to the TCA cycle (Fuchs et al., 2011). Within the genomes of *Azospirillum*, only *A. lipoferum* 4B and *Azospirillum* sp. B510 strain harbor the complete *paa* catabolic cluster; 11 of these genes are located on the p4/d replicon, whereas *paaX*, *paaY*, and *paaF* (which is duplicated) are located onto the chromosome, thus constituting five different clusters, an organization previously reported in *Pseudomonas putida* (Luengo et al., 2001). Seven of the genes located on p4 cluster have been classified as horizontally transferred, whereas the four others have been classified as ancestral (Wisniewski-Dyé et al., 2011), suggesting a complex evolution of this catabolic pathway. The functionality of the *paa* genes was confirmed as both strains were able to grow on phenylacetic acid as the sole carbon source, whereas *A. brasilense* strains showed no growth (Wisniewski-Dyé et al., 2012).

Thus, *A. lipoferum* 4B and *Azospirillum* sp. B510 seem to be more versatile for aromatic compound degradation than *A. brasilense* strains; indeed, in addition to the aforementioned pathways, several aromatic ring-hydroxylating dioxygenases could be identified. Whether this versatility is related to the composition of the host plant exudates and is a result of niche-specific adaptation remains to be elucidated.

25.1.5.3 Response to Stress and Iron Acquisition. *Azospirillum* must cope with reactive oxygen species (ROS) produced by plants, as a defense mechanism against both pathogenic and symbiotic bacteria (Lamb and Dixon, 1997; Santos et al., 2001). Comparative genomic analysis shows that next to common mechanisms, several enzymes involved in the oxidative stress response differ among *Azospirillum* species. A gene encoding a superoxide dismutase (SodA) is found uniquely in *A. brasilense* strains, whereas a gene encoding a catalase is present in *A. lipoferum* 4B and in *Azospirillum* sp. B510. Moreover, a bifunctional catalase-peroxidase (KatG) is only found in *A. lipoferum* 4B and displays 80% identity with KatG of *Rhizobium etli*, known to play a role in survival during stationary phase (Vargas et al., 2003). The genome of *Azospirillum* sp. B510 also harbors extra genes encoding glutathione-*S*-transferases, two being classified as HGT (AZL_014610 and AZL_e01570); such enzymes can detoxify a large variety of compounds by covalent linking of glutathione to hydrophobic substrates (Vuilleumier and Pagni, 2002). Having endophytic properties, this strain can be more exposed to plant secondary metabolites and such enzymes could confer the ability to cope with those compounds.

Several open reading frames (ORF)s in all four *Azospirillum* sequenced genomes have been annotated as laccase-like. Laccases- or laccase-like multicopper oxidases (EC 1.10.3.2) catalyze the oxidation of various substrates, such as phenols, diamines, and metals, coupled with the reduction of molecular oxygen to water. Bacterial laccases are involved in various functions such as copper resistance,

manganese oxidation, pigmentation, oxidation of toxic compounds, and destruction of ROS and are considered as an advantageous trait for a rhizosphere bacterium (Sharma et al., 2007). However, only one ORF (AZOLI_p30139 in *A. lipoferum* 4B and AZL_c02540 in *Azospirillum* sp. B510) possesses two typical copper-binding motifs (Kellner et al., 2008). Interestingly, the first report of a prokaryotic laccase is from *A. lipoferum* 4B (Givaudan et al., 1993), where it was shown to play a role in melanization and utilization of plant phenolic compounds (Faure et al., 1994). Moreover, laccase-positive strains are less sensitive to the inhibitory action of quinone analogs due to rearrangements of their respiratory chain, a feature that might be a competitive advantage in the rhizosphere in the presence of quinone compounds (Alexandre et al., 1999).

Discrepancies between *Azospirillum* strains are observed for iron acquisition, at the level of siderophore biosynthesis and uptake, and could be relevant as plant colonization ability is linked to iron acquisition systems (Molina et al., 2005). A 14 kb region, predicted to be involved in pyochelin biosynthesis, appeared to be exclusively present in the *A. lipoferum* 4B genome. The corresponding genes, classified as HGT, show protein identity levels greater than 45% and a high degree of synteny with those of the strain *Pseudomonas fluorescens* Pf-5. A cluster of genes involved in enterobactin biosynthesis was specifically found in the two *A. brasilense* strains (Sp245 and CBG497).

25.1.5.4 Type VI Secretion System. Components of a type VI secretion system (T6SS) have been identified in the four assembled published genomes and classified as HGT; however, the T6SS components display discrepancies among strains (Wisniewski-Dyé et al., 2011; 2012). T6SS are involved in a broad variety of bacterial functions: pathogenesis (by delivering effectors to target eukaryotic cells), biofilm formation, stress sensing, and toxicity toward other bacteria (Bernard et al., 2010; MacIntyre et al., 2010). Upregulation of *A. brasilense* Sp245 T6SS genes in response to exposure to IAA, as could happen in the rhizosphere, is in favor of a role in plant–bacteria interactions (Van Puyvelde et al., 2011).

25.2 CONCLUDING REMARKS

The availability of *Azospirillum* and host plant genomes offers new perspectives; indeed, transcriptome and functional analyses can now be carried out in order to characterize key genetic determinants involved in the *Azospirillum*–plant association and to elucidate the molecular bases underlying its specificity. Furthermore, the access to new *Azospirillum* genomes should provide important clues to evolutionary dynamics and events that lead to specific adaptation to a host plant.

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Chapter 26

Pangenome Evolution in the Symbiotic Nitrogen Fixer *Sinorhizobium meliloti*

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26.1 INTRODUCTION

The species concept is challenged by genomes of certain bacterial species: in fact, in many cases the genomic variability at the level of many bacterial species has led to a new definition of the so-called "pangenome" (Medini et al., 2005). In particular, the pangenome of a species is composed of a "core" fraction conserved in all the strains and a "dispensable" fraction present only in a strain(s) subset; the dispensable genome can be additionally divided into "accessory" and "unique," the latter comprising of only the single strain-specific genomic regions. In most bacterial species, the dispensable genome has a size comparable to the core fraction, thus complicating the definition of the bacterial species (Gogarten and Townsend, 2005; Kislyuk et al., 2011). The genes encoded by the dispensable genome fraction are thought to account for the adaptation of each strain to different niches and are most likely being originated by relatively recent horizontal gene transfer (HGT) events (Medini et al., 2008).

The ratio between the core and dispensable fraction in the pangenome of a species is also useful in determining the lifestyle(s) of that species. In fact, pangenomes can be classified either as "open" or "closed" by looking at the pangenome growth curve; "open" pangenomes have virtually no upper limit to the number of genes in the pangenome, while for "closed" pangenomes this limit is reached within a limited number of strains. Open pangenomes belong to species able to live in different ecological niches and being more exposed to HGT events. On the contrary the closed pangenomes are typical of pathogens and obligate symbionts (Tettelin et al., 2008). The pangenome broad structure is therefore useful in determining the general lifestyle of a species without the need to look at gene annotations.

Another important factor for the determination of a species lifestyle is the presence of additional replicons

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other than the chromosome. In fact, many bacterial species possess additional replicons with sizes comparable to the chromosome, called "megaplasmids"; a particular kind of megaplasmids, harboring core genes and having plasmid-like partitioning systems, have been recently defined as "chromids," showing that they are genera-specific elements, whereas the megaplasmids, being more recent in evolutionary terms, may be used to define the species boundary inside genera (Harrison et al., 2010;). In many bacterial species it has been shown that megaplasmids and chromids have rather specific functional signatures, such as the symbiotic megaplasmids in rhizobial species harboring most of the genes needed for an effective symbiosis (Barnett et al., 2001; Reeve et al., 2010), their presence being therefore strongly related with the species lifestyle. The yet unknown role of the different replicons (chromosome, chromids and megaplasmids) in the microevolution of bacterial species has been recently elucidated in *Sinorhizobium meliloti* in two distinct studies (Epstein et al., 2012; Galardini et al., 2013). This alphaproteobacterium is a nitrogen fixer symbiont of *Medicago* plants and has been an ideal model system to understand bacterial microevolution and the role of each replicon in intraspecies differentiation. From an ecological point of view, *S. meliloti* is found as a free-living organism in temperate soils around the globe, as a symbiont in root nodules of *Medicago* plants, and as an endophyte in several plant species (Chi et al., 2005; Pini et al., 2012). The genome of the *S. meliloti* type strain Rm1021, the first to be sequenced, possesses a chromosome, a chromid (pSymB), and a megaplasmid (pSymA) (Galibert et al., 2001); the pSymA megaplasmid contains most of the genes needed to establish an effective symbiosis and other genes related to nitrogen metabolism (Barnett et al., 2001), while the pSymB chromid is mostly deputed to support the saprophytic phenotype of the species in the soil environment and it contains many important core genes; for this reason it has been classified as a chromid (Finan et al., 2001; Harrison et al., 2010). Such functional diversification has been found appropriate also in other sequenced *S. meliloti* strains (Galardini et al., 2011; Schneiker-Bekel et al., 2011); moreover, the presence of conserved core genes on the pSymB chromid leads to the hypothesis that the chromosome and the pSymB chromid may be genus specific and relative more ancient than the megaplasmid pSymA, which may be selected during strain differentiation.

This chapter discusses the recent findings on the pangenome of the *S. meliloti* species, focusing not only on the core/dispensable fraction in the whole pangenome, but also on their role in each replicon in the context of species microevolution (Epstein et al., 2012; Galardini et al., 2013; Sugawara et al., 2013).

26.2 THE OPEN PANGENOME OF *S.meliloti* AGREES WITH THE SPECIES LIFESTYLE

The pangenome of a panel of 14 *S. meliloti* strains has been recently analyzed in a study aimed at determining whether the pangenome of this species was open or closed (Galardini et al., 2013). The general features of these *S. meliloti* genomes are outlined in Table 26.1: the overall genome size varies across the strains, from the 6.69 Mb of strain Rm1021 to the 8.94 Mb of strain 5A14, while the GC content shows a lower variability, ranging from 61.3% in strain Rm1021 to 62.0% in strains 5A14, AE608H and BL225C. The gene density appears to be conserved as well across the strains, even though with a higher variability, ranging from 82.53% in strain AK83 to 86.40% in strain SM11.

As expected, the pangenome of the *S. meliloti* species is open, in accordance to the species free-living and in association with plants either through symbiosis or endophytism lifestyles. The pangenome rarefaction curve (Fig. 26.1) suggests that the dispensable genome size increases with an almost constant rate with the addition of new genomes to the dataset, while the core genome size decreases with a slower rate: in particular, the accessory component of the dispensable genome grows faster than the unique component.

The pangenome of other 33 *S. meliloti* strains has also been determined recently, showing a relatively similar core genome size (4680 OGs, against the 3989 found in the 14

Table 26.1 General Features of the *Sinorhizobium meliloti* Strains Used in This Study

	1A 42	5A 14	A06 41M	A064 3DD	AE6 08H	AK11	AK75	C04 31A	C043 8LL	H1	Rm1021	AK83	BL2 25C	SM11
Source	Iran	Iran	Italy	Italy	Italy	Kazhakstan	Kazhakstan	Italy	Italy	Italy	—	Kazhakstan	Italy	Germany
Length (Mb)	7.16	8.94	7.95	7.35	7.35	6.84	6.99	7.09	7.06	6.92	6.69	7.14	6.98	7.5
G + C content	62.02	61.98	61.88	61.84	62	62.03	61.86	61.96	61.96	61.96	61.30	61.90	62.00	61.91
Coding %	85.25	85.57	85.17	85.28	85.46	85.75	85.28	85.2	85.44	85.11	86.13	82.53	84.56	86.4
ORFs	7374	8735	8411	7771	7197	6895	7555	7386	7242	6993	6218	6518	6359	7428

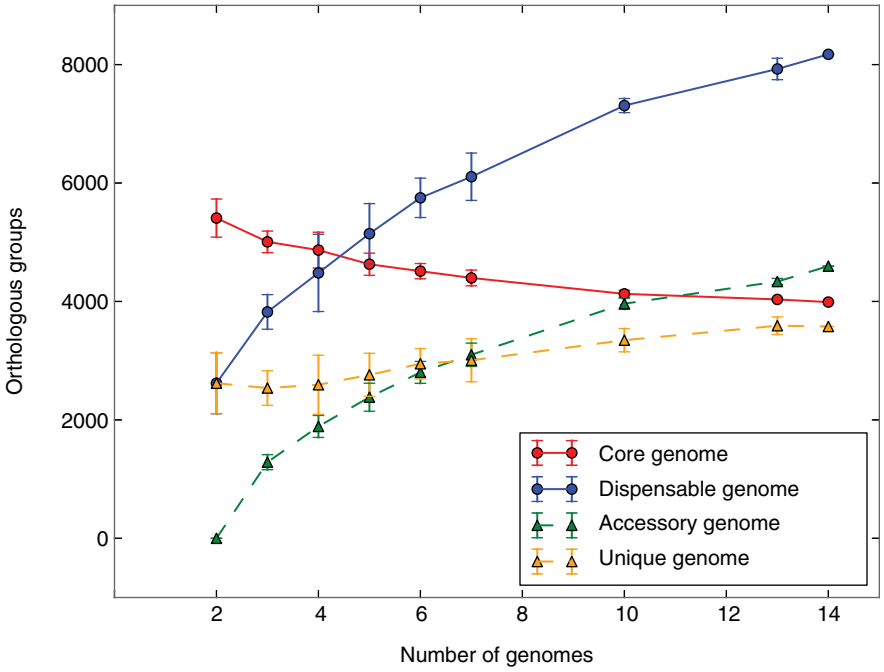

Figure 26.1 *S. meliloti* pangenome rarefaction curves.

strains analysis) and also a similar variability in the unique genome size for each strain (varying from 25 to 840 OGs) (Sugawara et al., 2013); the pangenome rarefaction curve has not been computed for these strains, but the similar size of the core and unique genome suggests that it may be similar to the one computed for the other 14 strains.

26.3 REPLICON DIVERSITY: THE CHROMID HAS AN IMPORTANT ROLE IN INTRASPECIES DIFFERENTIATION

Both the pSymB chromid and the pSymA megaplasmid have a higher genetic diversity in the *S. meliloti* pangenome than the chromosome. This feature has been observed when comparing 24 *S. meliloti* strains against the reference genome of strain Rm1021, where the average nucleotide diversity is found to be 6.1 and 3.6 times greater than the one observed in the chromosome for the pSymA megaplasmid and for the pSymB chromid, respectively. As expected, the chromosome is found to be the most conserved replicon at the nucleotide level, but with two distinct levels of nucleotide conservation, higher for the first half of the replicon (from the origin to the replication terminus) and lower for the second half. This lower nucleotide variability among the analyzed strains may be related to a putative selective sweep on the second half of the chromosome (Epstein et al., 2012). This finding suggests that the chromosome controls the genetic hitchhiking (at least on part of the replicon), thus reducing intraspecies variability and ensuring a more strictly vertical inheritance.

Given the stability of the chromosome, the question of which replicon contributes the most to intraspecies differentiation remains open for either the pSymA megaplasmid or the pSymB chromid. To answer this question, the core and dispensable genome fractions analyzed by Galardini and colleagues (Galardini et al., 2013) were divided according to the replicon of origin. The genome structure of each strain was estimated by mapping the draft contigs against the complete genomes. As indicated in Table 26.2, in all the draft genomes the replicon sizes were conserved; the replicon-specific dendrograms suggest that the pSymB chromid may be the replicon that most contributes to the intraspecies differentiation in *S. meliloti*, in both the core and accessory pangenome fractions, showing the highest number of clusters (Fig. 26.2).

On the other hand, the pSymA megaplasmid shows the same strain composition in each genome fraction, showing also geographical clusterization for some strains; both findings suggest a more recent evolutionary origin for this replicon.

26.4 ANCIENT VERSUS RECENT: DIFFERENT TIMING OF HGT EVENTS

Genes in the *S. meliloti* pangenome show different taxonomic homologies with respect to their replicon of origin. When looking at the similarity with other bacterial species from different phyla, genes from the pSymB chromid appear to have a bigger homology signal to proteins belonging to β and γ *proteobacteria* classes than genes belonging to the chromosome and the pSymA megaplasmid (Fig. 26.3).

Table 26.2 Real and estimated replicons size (in Megabases) in the 14 *S. meliloti* strains

	1A42	5A14	A0641M	A0643DD	AE608H	AK11	AK75	C0431A	C0438LL	H1	Rm1021	AK83	BL225C	SM11
Chromosome	3.73	4.99	4.06	3.64	3.89	3.57	3.45	3.61	3.59	3.56	3.65	3.82	3.67	3.91
Chromid pSymB	1.59	1.88	1.75	1.59	1.63	1.57	1.65	1.56	1.63	1.60	1.68	1.68	1.69	1.63
Megaplasmid pSymA	1.40	1.51	1.46	1.31	1.29	1.39	1.52	1.39	1.31	1.37	1.35	1.31	1.61	1.63
pSINME01	0.21	0.23	0.11	0.14	0.13	0.11	0.08	0.11	0.09	0.13	—	0.26	—	—
pSINME02	—	—	—	0.01	—	0.03	—	—	0.01	—	—	0.07	—	—
pSmeSM11b	0.14	0.18	0.06	0.12	0.13	0.01	0.03	—	0.07	0.01	—	—	—	0.18
pSmeSM11a	0.01	—	—	0.23	—	0.02	—	0.01	—	—	—	—	—	0.14
Not mapped	0.08	0.16	0.50	0.30	0.28	0.15	0.27	0.40	0.36	0.25	—	—	—	—

The replicons of strains Rm1021, AK83, BL225C and SM11 are known from their complete genomic sequence, while for the other strains the average mapped bases to each replicon of the four complete genomes are indicated.

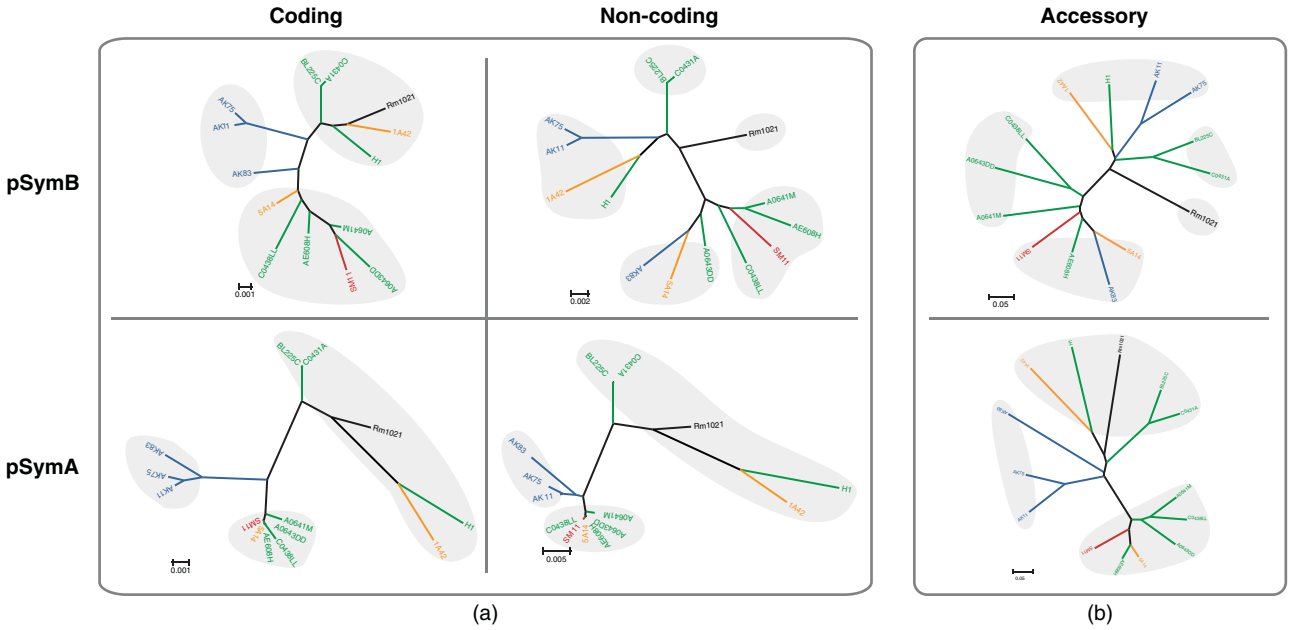

Figure 26.2 Dendrograms of the pangenome content with respect to the pSymB chromid and the pSymA megaplasmid.

Genes belonging to the pSymB chromid have higher phylogenetic similarities than the other replicons also for the phyla *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Fibrobacteres,* and *Firmicutes* (Galardini et al., 2013). This broad range of phylogenetic similarities of chromid genes may indicate that a significant fraction of the pSymB chromid has originated by relatively ancient HGT events and may therefore have contributed to the emergence of a new taxa such as the *Sinorhizobium* genus (Harrison et al., 2010).

On the other hand, more recent HGT events with the near *Sinorhizobium medicae* species are more common on the pSymA megaplasmid (77.3% of all the predicted HGT events), while the chromosome shows only one gene transferred through HGT, thus confirming the selective control on this replicon (Epstein et al., 2012). Moreover, many of the genes being transferred through HGT between *S. meliloti* and *S. medicae* are grouped in clusters, confirming that megaplasmid pSymA evolutionary history may be mainly due to recent gene transfer events.

26.5 REPLICON-SPECIFIC FEATURES OF THE *S.meliloti* PANGENOME

Each replicon of the *S. meliloti* pangenome (partially) acts as an independent evolutionary unit and has a distinct role in both functional and evolutionary aspects. In particular, the chromosome is mainly composed by core genes with a limited variability between strains and whose function is mostly related to housekeeping functions; the lower variability and low number of HGT events (both from/to distant taxa and near species) indicate that this replicon

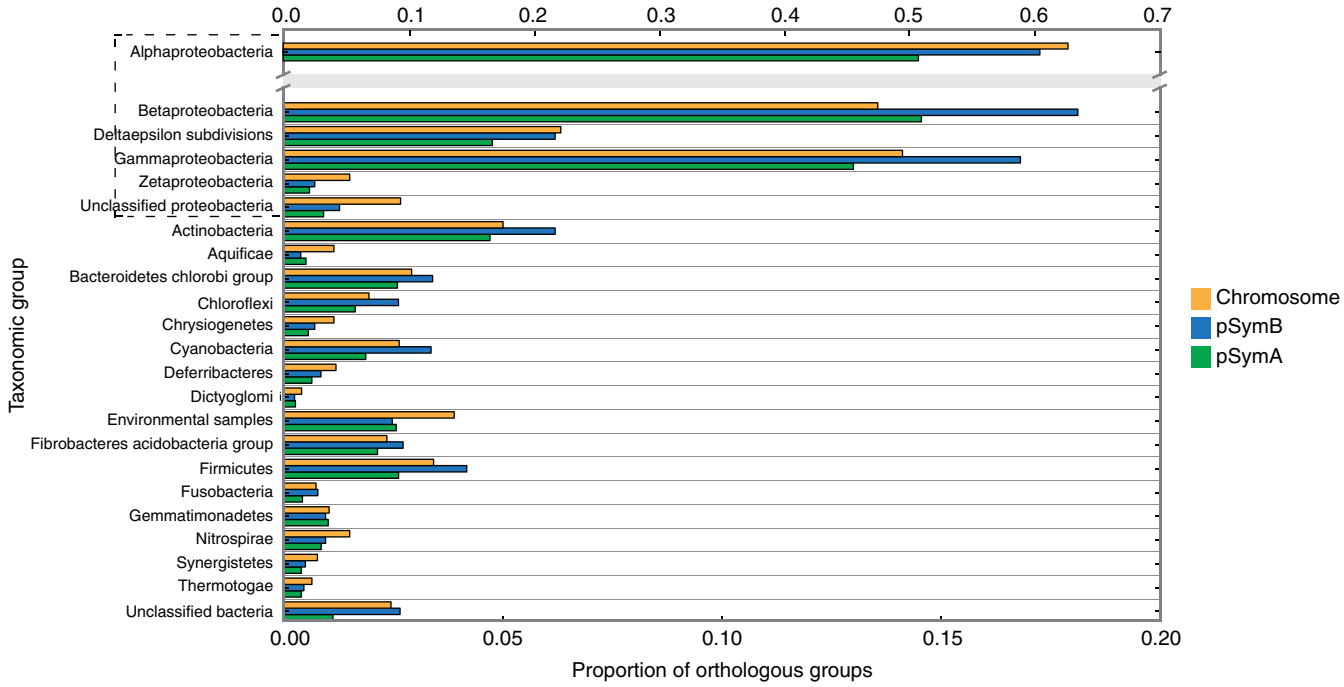

Figure 26.3 Taxonomic distribution of OGs belonging to different replicons.

evolves mostly through vertical inheritance. The pSymA megaplasmid harbors most of the genes needed to establish an efficient symbiosis (Barnett et al., 2001) and contains mostly dispensable genes; the similar phylogenetic pattern observed for both core and dispensable genes suggests that most of the gene content of this replicon may have originated by relatively recent HGT events, a feature confirmed by the higher number of genes transferred from/to *S. medicae* than the other replicons. Unexpectedly, the role of the pSymB chromid appears to be related to species differentiation; whether this role is found also in other species or at other taxonomical levels is unknown and yet to be established. The higher homology of its genes to distant bacterial taxa, compared to the other replicons, indicates that this replicon most probably has originated by relatively ancient HGT events that may be at the root of the genus *Sinorhizobium*.

26.6 CONCLUSIONS AND FUTURE PERSPECTIVES

The traditional view of bacterial genomes, composed by one chromosome and optionally several small plasmids does not take into account many bacterial species, often associated with multiple diverse lifestyles, such as the facultative plant symbiont *S. meliloti*. The peculiar characteristics of each of the three *S. meliloti* replicons have been analyzed for their functional features (Barnett et al., 2001; Finan et al., 2001; Harrison et al., 2010). However, until recently, the analysis of the evolutionary and phylogenetic role of each replicon has been missing. Here, we have discussed recent studies showing that the chromosome is the most conserved replicon of the *S. meliloti*, while the pSymA megaplasmid has a higher variability but a phylogenetic coherence between core and dispensable fraction that suggests recent HGT events; on the contrary, the pSymB chromid appears to drive the intraspecies differentiation and may originate at the *genus* level from ancient HGT events.

These replicon-dependent features of the *S. meliloti* species may also be shared by other bacterial species with open pangenomes and multipartite genomes, which are very common among rhizobial species. Similar studies on different species may confirm these observations and open the way to the definition of new rules describing the evolution of complex bacterial genomes, with the chromosome as a stable "container" of core functions, the chromid as an important source of genus-specific functions and intraspecies variants, and the megaplasmid as a fluid container of specific functions related to lifestyles.

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Chapter 27

Pangenomic Analysis of the *Rhizobiales* Using the GET_HOMOLOGUES Software Package

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27.1 INTRODUCTION

The advent of next-generation sequencing (NGS) technology has recently boosted the number of genome sequencing projects publicly available (Pagani et al., 2012). This trend is also observed in the *Rhizobiales,* an order of the *Alphaproteobacteria*-containing bacteria of contrasting ecologies and lifestyles. They exhibit a wide range of genome sizes, ranging from <1.2 to >8.8 Mb. Different genome architectures can be found among the *Rhizobiales*. Some contain large megaplasmids >1.5 Mb, which carry essential core genes such as chromosomes, but replicate via repABC-like systems (Landeta et al., 2011). The term "chromid" has been suggested for this type of replicons (Harrison et al., 2010). Others carry secondary linear chromosomes that show lower proportion of shared genes and lower levels of synteny (Slater et al., 2009). The lifestyles of these bacteria comprise facultative intracellular vertebrate pathogens (*Brucella*, *Bartonella*), facultative N2-fixing root-nodule bacteria or plant pathogens (*Agrobacterium*, *Azorhizobium*, *Bradyrhizobium*, *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*), photosynthetic bacteria such as *Rhodopseudomonas*, or methylotrophs such as *Methylobacterium*, to mention but a few. Many of these bacteria establish long-term, often chronic infections of hosts, ranging from pericellular colonization (*Agrobacterium*) to facultative intracellular multiplication such as root nodule bacteria (rhizobia) or *Brucella* spp. (Batut et al., 2004). Therefore, these bacteria represent an ecologically and genomically heterogeneous group of organisms exhibiting an abundance of adaptations (Ettema and Andersson, 2009). For example, *Rhodopseudomonas palustris* has the ability to switch between the four different modes of metabolism that support life: photoautotrophic, photoheterotrophic, chemoautotrophic, and chemoheterotrophic (Larimer et al., 2004). Other genomes, such as those of the *Bartonellaceae*, are small and show limited metabolic diversity. They show evidence of genome reduction as an adaptation to host-associated intracellular lifestyles (Sallstrom and Andersson, 2005). The abundance of whole genome sequence data currently available for the *Rhizobiales* represents a powerful resource for comparative genomics to study the evolution of host–microbe interactions and niche adaptation.

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A fundamental necessity for such studies is the inference of orthology relationships among the proteomes under analysis (Fitch, 1970; Koonin, 2005; Sonnhammer and Koonin, 2002). The correct inference of orthologous groups is critical in phylogenetic reconstruction, genome annotation, structural genomics, and experimental target selection, among others. Orthologous genes have diverged from their common ancestors by a speciation event and tend to maintain their functionality across large evolutionary distances, while paralogs tend to diverge over time via sub-functionalization or neo-functionalization pathways, thus acquiring new functions (Koonin, 2005). Xenology is the homology relationship resulting from horizontal gene transfer (HGT), which is a major force driving genome evolution in microbes (Gogarten and Townsend, 2005; Treangen and Rocha, 2011). Many microbial species, both eukaryotic and prokaryotic, have a mosaic genome structure, consisting of conserved syntenic regions alternating with highly divergent genomic islands acquired by HGT (Dobrindt et al., 2004; Rolland et al., 2009; Welch et al., 2002; see Chapters 25, 26), which generally insert into the recipient genome via site-specific recombination. A well-characterized island in the *Rhizobiales* is the symbiotic island of *M. loti* (Sullivan et al., 1995; Sullivan and Ronson, 1998; see Chapter 21). It was the seminal comparative genomics work of Tettelin and colleagues on multiple *Streptococcus agalactiae* strains (Tettelin et al., 2005) that revealed that this species "can be described by a pangenome consisting of a core genome shared by all isolates, accounting for approximately 80% of any single genome, plus a dispensable genome consisting of partially shared and strain-specific genes". A similar pangenome structure has been observed in many other bacterial groups, making the terms core- and pangenome common in microbial comparative gemonics (Koonin and Wolf, 2008; Medini et al., 2005; Rasko et al., 2008). The core genome genes generally encode essential genes of the central metabolism, replication, transcription and translation machinery, as well as DNA recombination and repair functions. Orthologous core genome genes are the most suitable for inferring species phylogenies or estimating population genetics parameters, as they convey the information of speciation events, tend to be selectively neutral, and are less prone to HGT (Vinuesa, 2010). Genes in the dispensable genome are frequently found associated with mobile genetic elements and often encode key adaptive traits for specific niches or habitats, defining the unique phenotypic attributes of particular groups of strains (Gogarten and Townsend, 2005; Medini et al., 2005; Tettelin et al., 2008; Galardini et al., 2011; Rasko et al., 2008; see Chapter 25).

We have recently developed a computationally efficient, versatile, and user-friendly bioinformatic pipeline for microbial pangenomics and comparative genomics, which we refer to as "GET_HOMOLOGUES" (Contreras-Moreira and Vinuesa, 2013). In this chapter we illustrate some of its features by performing several pangenomic analyses on 68 representative and completely sequenced *Rhizobiales*. We first compute robust consensus core- and pangenome sets of homologous gene families and estimate the size of these genome fractions. Whole genome phylogenies are inferred on the basis of either the conserved core genome, or the pangenome phylogeny, based on the presence–absence matrix of homologous gene families across genomes. The results are discussed in broad genomic, ecological, and evolutionary contexts.

27.2 METHODS

GET_HOMOLOGUES is an open source software package released under the GNU license, written in Perl (http://www.perl.org/), and R (R Development Core Team, 2012) for the automatic, customizable, and efficient computation of clusters of homologous gene families and downstream pangenomic and comparative genomics analyses on UNIX and Linux machines. It builds upon popular orthology-calling approaches based on heuristic pair-wise best-match methods (Altenhoff and Dessimoz, 2012; Kristensen et al., 2011). The input sequences can be either in FASTA (proteomes) or GenBank formats. Sequences are automatically extracted and the proteomes subjected to pair-wise BLAST+ (Camacho et al., 2009) analysis. The main script *get_homologues.pl* can cluster BLAST results using our own implementation of the bidirectional best-hit (BDBH) algorithm (Overbeek et al., 1999; Tatusov et al., 1997), or by calling the COGtriangles (Kristensen et al., 2010) or OrthoMCL (OMCL) (Li et al., 2003) clustering algorithms. Table 27.1 provides a short description of the main features of these algorithms.

The stringency of the resulting clusters of homologous proteins can be fine-tuned by controlling certain parameters such as E-value cut-off, pair-wise alignment coverage cut-off, and/or by scanning the Pfam (Finn et al., 2008) domain composition of proteins using hmmscan from the HMMER3 package (Eddy, 2009). A unique feature of GET_HOMOLOGUES is that clusters of homologous gene families can be made very robust by computing consensus clusters from those generated by any combination of the clustering algorithms and filtering criteria mentioned above. Several auxiliary scripts are provided to download selected genomes from NCBI and extract sequences in FASTA format (*download_genomes_NCBI.pl*), compare, filter and interrogate clusters of homologous sequences (*compare_clusters.pl*), compute and graphically display the core and pangenome sets, and perform comparative genomics analyses and (*plot_pancore_matrix.pl* and *parse_pangenome_matrix.pl*). The exponential (Tettelin et al., 2005) and binomial mixture models (Snipen et al., 2009) can be fitted to the data to estimate theoretical core and pangenome sizes, and high quality graphics

Table 27.1 Description of the clustering algorithms implemented in GET_HOMOLOGUES

Algorithm	Description
BDBH	Starting from a reference genome, keep adding genomes stepwise while storing the sequence clusters that result of merging the latest bidirectional best hits.
COGtriangle	Merges triangles of intergenomic symmetrical best matches, as described in (Kristensen et al., 2010). Note that a single sequence might occasionally be included in several COGS clusters with option −x.
OMCL	OrthoMCL v1.4, uses the Markov Cluster Algorithm to group sequences, with inflation (−F) controlling cluster granularity, as described in Li et al. (2003).

generated by calling R functions. Pangenome trees can be computed from pangenomic presence–absence data using the parsimony optimality criterion as implemented in pars from the PHYLIP package (Felsenstein, 2004). Basic comparative genomics analyses can be performed to identify lineage-specific genes or gene family expansions. The software is designed to take advantage of modern multiprocessor personal computers or servers, as well as computer clusters running the open source batch-queuing system Grid Engine, to parallelize time-consuming BLAST and hmmscan runs. Large genome sets can be analyzed even on machines with modest RAM resources (<10 GB) by invoking the BerkeleyDB option, which trades RAM usage for CPU usage, and/or by invoking a heuristic BDBH version that minimizes the number of BLAST searches between proteomes (Contreras-Moreira and Vinuesa, 2013). The clusters of homologous gene families are output in standard multi-FASTA format, or in an extended OrthoXML format (Schmitt et al., 2011), to facilitate exchange of orthologous sequence data, in compliance with recent community standards (Dessimoz et al., 2012). The software package is available for 32 and 64 bit processors on Linux and Mac OS X machines. It includes a user manual with detailed hands-on tutorials, and an installation script that checks for optional software dependencies and guides the user on how to proceed to install them. This script also supports downloading and formatting the current version of Pfam. The latest version of the package can be downloaded at http://www.eead.csic.es/compbio/soft/gethoms.php and http://maya.ccg.unam.mx/soft/gethoms.php.

27.3 RESULTS AND DISCUSSION

27.3.1 Computation, Graphical Display, and Statistical Analysis of the Core and Pangenome of 68 *Rhizobiales*

A total of 68 genomes of the *Rhizobiales* were downloaded from GenBank's FTP site using the auxiliary script *download_genomes_NCBI.pl*, bundled with the GET_HOMOLOGUES package. The main script *get_homologues.pl* was called under default settings BlastP Expectation value: −E 0.00001; alignment coverage: −C 75; reporting all clusters: −t 0, OMCL inflation parameter: −F 1.5] to compute families of homologous gene sequences with each of the three clustering methods implemented in GET_HOMOLOGUES (Table 27.1). As summarized by the Venn analysis shown in Figure 27.1a, each clustering method produced notoriously different results, as expected from their algorithmic differences, some of which are explained below. A unique feature of GET_HOMOLOGUES is the possibility to compute consensus clusters, that is, to filter out those families consistently recovered by all or any combination of the three clustering methods (Table 27.1). Such clusters are therefore very robust, although highly conservative. In the analysis presented in Figure 27.1a, a consensus core-genome consisting of 323 gene families was computed out of the intersection of the three algorithms for the 68 *Rhizobiales* genomes analyzed. Note that the COGtriangles method computes the largest set of unique clusters (Fig. 27.1a). This is due to the fact that many of them are singletons, that is, clusters containing less than three members. By definition, the COGtriangles algorithm requires at least three proteins in different species to form a cluster (Kristensen et al., 2010; Tatusov et al., 1997). On the other extreme, the BDBH algorithm produced the lowest number of unique clusters. This is expected since BDBH has the limitation of requiring the presence of a homologous sequence in the reference genome to consider the cluster (Contreras-Moreira and Vinuesa, 2013).

This type of consensus analysis can also be applied for the pangenome. We computed an intersection of 14,297 clusters out of those generated by the COGtriangles and OMCL algorithms (Fig. 27.1b). These do not require a reference genome, as opposed to the BDBH algorithm, and hence are better suited for pangenome analyses than the latter. These core- and pangenome consensus clusters were generated using the auxiliary script *compare_clusters.pl* from the output generated by *get_homologues.pl*. The former script also generates the Venn analysis and diagrams depicted in Figure 27.1a and b.

Exponential decay or binomial mixture models can be fitted to the core-genome cluster data with the aid of the auxiliary script *plot_pancore_matrix.pl* based on the core_genome.tab matrix files generated by *get_homologues.pl*.

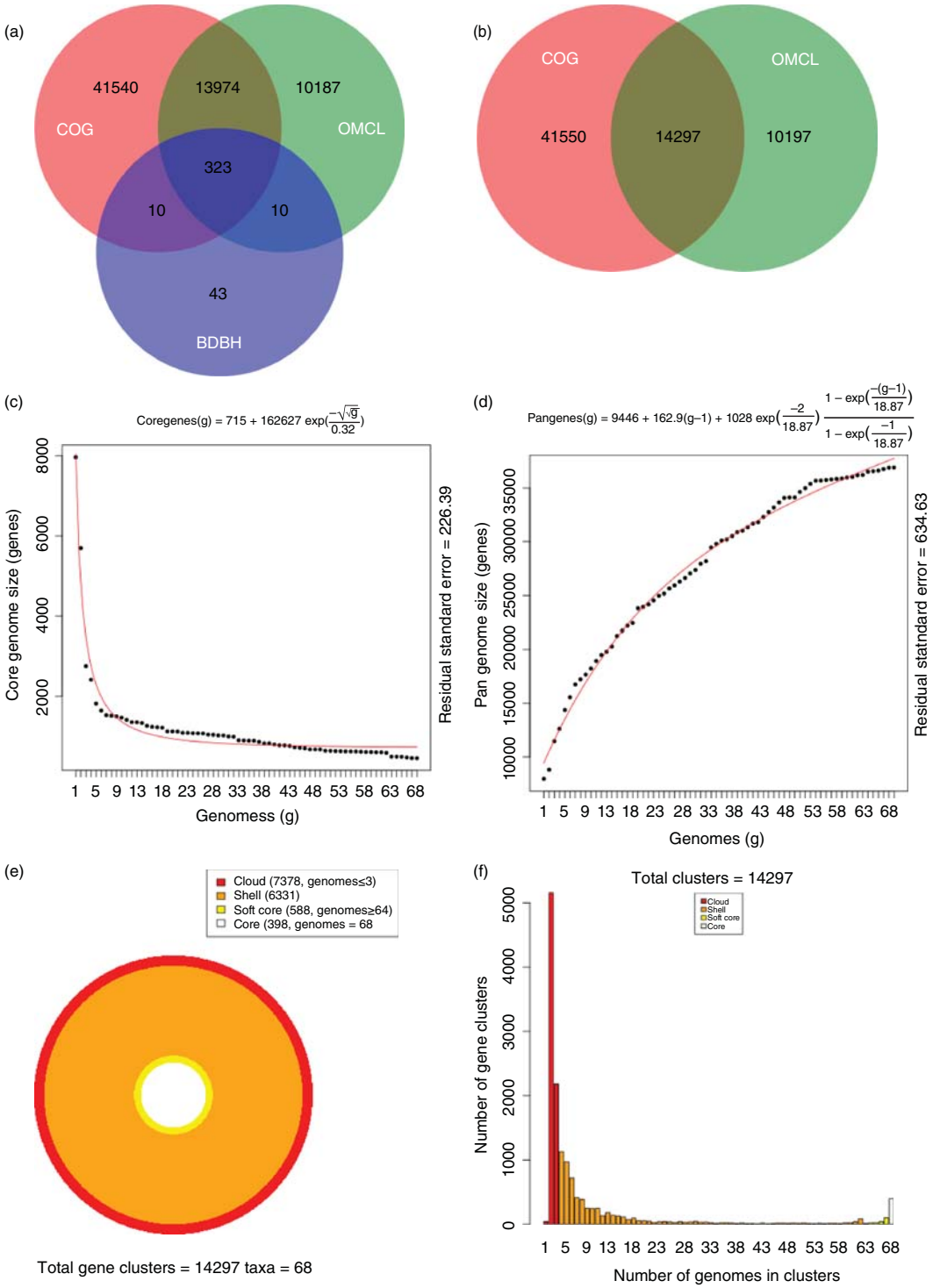

Figure 27.1 Pangenome analysis of 68 fully sequenced *Rhizobiales* genomes. (a) Venn analysis of the clusters generated by the BDBH, COGtriangles, and OrthoMCL algorithms for BLASTP results with E-value <10E − 5 and alignment coverage >75%. This produces an empirical consensus core-genome of 323 gene families. (b) Venn analysis of the clusters generated by the COGtriangles and OrthoMCL, which results in a consensus pangenome of 14,297 homologous gene families. (c) Theoretical estimation of the core-genome size of the *Rhizobiales* based on a Willenbrock model fit to OrthoMCL clusters, resulting in 715 gene families. (d) Tettelin fit to OrthoMCL clusters to estimate the size of the *Rhizobiales* pan-genome. (e) Circle plot of the observed sizes of the core, soft core, shell, and cloud genome fractions. (f) Distribution of cluster sizes as a function of the number of genomes they contain.

Figure 27.1c shows the plot of the exponential decay model of Willenbrock (Willenbrock et al., 2007) fitted to the core genome data for the OMCL algorithm. Notice that this function predicts a theoretical core genome with a size of 715 genes, which doubles the strict core size estimate made by the intersection of the three algorithms. This result highlights the complexities associated with theoretical core- and pangenome size estimations. The *plot_pancore_matrix.pl* script can also plot pangenome curves fitted with the exponential model proposed by Tettelin and colleagues (Tettelin et al., 2005). This analysis, run on the OMCL clusters, predicts that the theoretical pangenome of the *Rhizobiales* contains $\gg$40,000 genes, with $\sim$163 new genes being added on average to its pangenome with each new genome sequenced for bacteria from this order (Fig. 27.1d).

The auxiliary script *parse_pangenome_matrix.pl* is useful for both pangenome and basic comparative genomics analyses. Figure 27.1e shows a circle-plot showing the relative sizes of the strict and soft core-genomes, the shell-genome, and the cloud-genome compartments, as computed from the consensus clusters derived from the COGtriangles and OMCL algorithms. The soft-core is arbitrarily defined as the genes present in 95% or more of the genomes analyzed, as previously suggested by Kaas and colleagues (Kaas et al., 2012). The definition of a soft core genome is useful to get more robust core estimates when the dataset includes draft genomes, since those will, by necessity, miss some genes entirely, or contain truncated genes at the contig or scaffold ends. In our case study, 588 gene families were found in 64 or more genomes. The shell genome contains 6331 clusters present in >3 and <64 genomes. Finally, the most abundant class is that conformed by the so-called cloud genome, containing 7378 clusters in three or less genomes. Figure 27.1f shows the distribution of numbers of gene families as a function of their presence in 1–68 genomes. Such a distribution of the prokaryotic gene space was already observed in many other studies dealing with bacterial genomes at different levels of taxonomic depth, in which a minuscule fraction of genes is highly conserved in their phyletic distribution, while a much larger fraction (an order of magnitude in this case study of 68 *Rhizobiales*) is moderately conserved, and an even greater number of clusters is rarely encountered. In their seminal review on bacterial and archaeal genomics, Koonin and Wolf defined these components of prokaryotic gene space as the core, shell, and cloud compartments, respectively (Koonin and Wolf, 2008), which can be best described as the sum of three exponential decay functions. GET_HOMOLOGUES defines these compartments empirically, as follows: *Core* – genes contained in all considered genomes/taxa. *Soft core* – genes contained in 95% of the considered genomes/taxa, as in the work of Kaas and collaborators (Kaas et al., 2012). *Cloud* – genes present only in a few genomes/taxa. The cutoff was defined as the most populated non-core cluster class and its immediate neighboring classes. *Shell* – remaining genes, present in several genomes/taxa.

Snipen and colleagues criticized the use of exponential functions to estimate the sizes of the core and pangenomes (Snipen et al., 2009). This criticism stems from the fact that such models assume that the pangenome is composed of two types or classes of genes according to their phyletic distribution: a set of conserved core genes and a much larger set of dispensable genes. They extended the binomial mixture model originally proposed by Hogg and colleagues (Hogg et al., 2007), and found that for all genome sets analyzed in their study, the optimal mixture model has at least three components (Snipen et al., 2009). This is clearly in line with the above-mentioned study of Koonin and Wolf (Koonin and Wolf, 2008) and the results presented in Figure 27.1f. Such binomial mixture models are implemented in the *parse_pangenome_matrix.pl* script. The Bayesian information criterion indicates that for the 68 *Rhizobiales* dataset the optimal number of components for the mixture model is 8, producing an estimate of a theoretical core genome of size 270 and a pangenome of size 14,847 (Table 27.2).

Table 27.2 Binomial mixture model estimates of theoretical core- and pangenome sizes based on the consensus clusters generated by the COGtriangles and OrthoMCL algorithms

No. of Components	Core Size	Pan Size	BIC	Log Likelihood
2	402	14,300	256,835.864570013	−128,403.5805775
3	397	14,402	112,809.978810426	−56,381.0698927027
4	402	14,636	87,755.4665386494	−43,844.2459518106
5	397	14,765	81,400.9034621089	−40,657.3966085363
6	398	14,788	79,675.520958282	−39,785.1375516189
7	285	14,791	79,294.4986777406	−39,585.0586063441
8	**270**	**14,847**	**78,892.9034020882**	**−39,374.6931635139**
9	236	14,845	78,961.3328191793	−39,399.3400670554
10	262	14,838	78,916.2721469761	−39,367.2419259498

The row highlighted in bold indicates the best mixture model estimate, which corresponds to that with the lowest Bayesian information criterion value (BIC).

Particularly the estimate of the pangenome size seems too conservative when compared with the empirical observation of 14,297 consensus families estimated from the intersection of the COGtriangles and OMCL algorithms (Fig. 27.1b). However, the mixture model analysis and that presented in Figure 27.1e and f clearly show that the pangenome of the *Rhizobiales* has more than just two general categories of genes.

27.3.2 Phylogenomics of the *Rhizobiales* Based on a Supermatrix of the Core Genome

An important application of the consensus core genome clusters of orthologous gene families is their use in the phylogeny estimation of the order *Rhizobiales*. Each of the 323 families of the strict consensus core contains 68 sequences, one from each genome. These single copy orthologous gene families were individually aligned using muscle (Edgar, 2004) and the resulting alignments concatenated. The resulting supermatrix was then subjected to maximum likelihood phylogeny estimation using PhyML 3.0 (Guindon et al., 2010) under the LG model, with among-site rate variation estimated by fitting a gamma distribution with five rate classes, and empirical amino acid frequencies estimated from the data. The resulting core genome phylogeny for 68 *Rhizobiales* with midpoint rooting is shown in Figure 27.2.

Notice the high resolution of the phylogenetic hypothesis, as most bipartitions have Shimodaria-Hasegawa-like support values of 1. Two major clades are observed based on

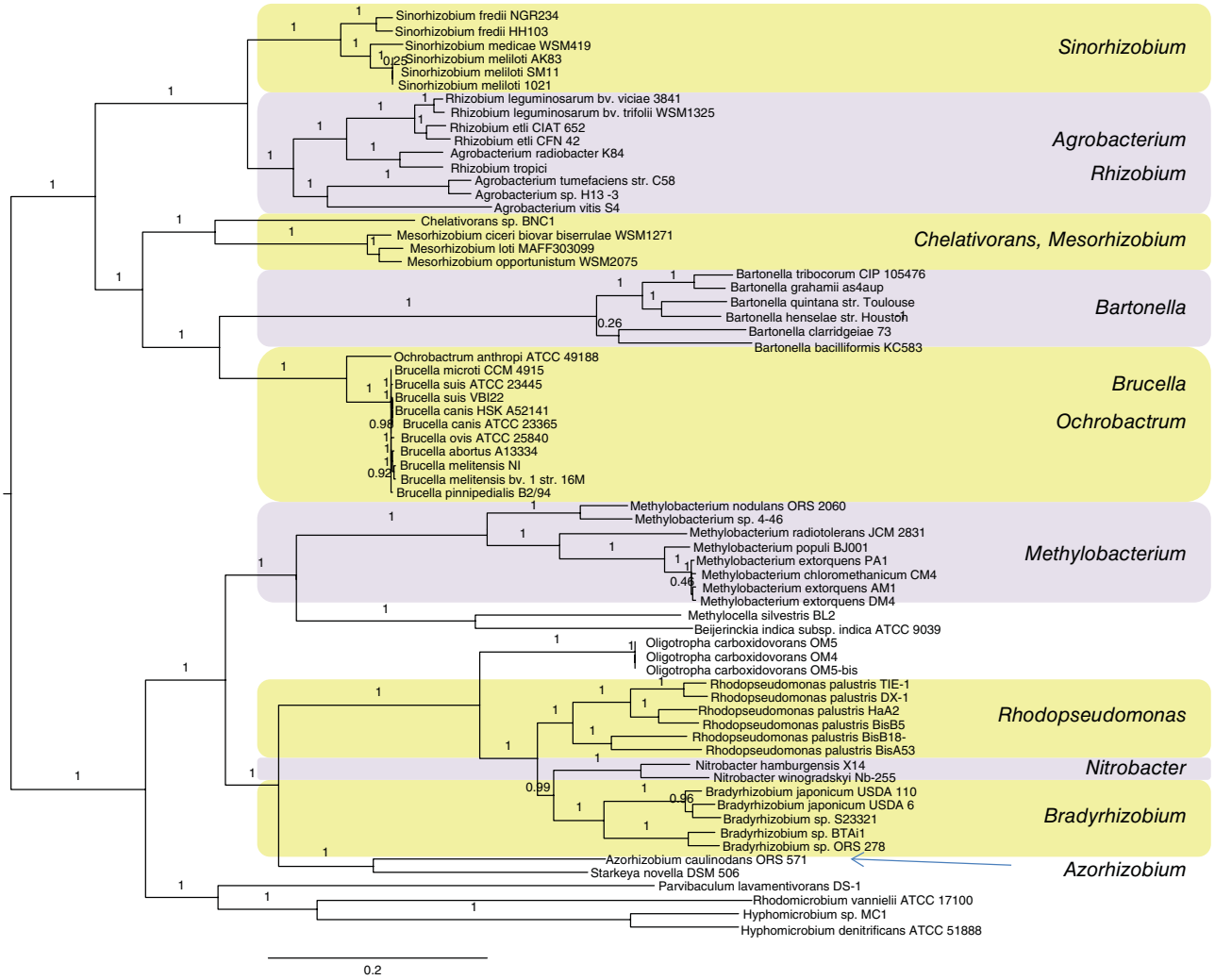

Figure 27.2 Phylogenomic analysis of the *Rhizobiales* based on a supermatrix of 323 consensus clusters of single copy orthologous genes derived from the intersection of the BDBH, COGtriangles, and OrthoMLC results. The phylogeny was estimated under the maximum likelihood criterion using the LG + G model with empirically estimated amino acid frequencies and rooted using midpoint rooting. The scale represents the estimated number of substitutions per site. Numbers on tree bipartitions indicate the Shimodaria-Hasegawa-like support values.

the midpoint rooting, which is consistent with the topology of a large genomic phylogeny for the Alphaproteobacteria (Williams et al., 2007). The first one groups genera in the families *Brucellaceae*, *Bartonellaceae, Phylobacteriaceae, and Rhizobiaceae*. They all form monophyletic entities, and therefore their classification in the corresponding families is consistent with the phylogenomic hypothesis presented in Figure 27.2. Two things are notorious in this clade: the long branch subtending the *Bartonella* cluster and the very limited genetic diversity found within the genus *Brucella* for the core genome loci. The former observation is typical of bacterial species undergoing genome reduction and degradation as a result of adaptation to intracellular lifestyle (Sallstrom and Andersson, 2005), while the latter is typical of clonal population structures, as reported for *Brucella* spp. based on multilocus sequence analysis (Whatmore et al., 2007).

The major groupings in the second clade correspond to the families *Bradyrhizobiaceae* and *Methylobacteriaceae*. These and the other families clustered in this clade are monophyletic, which again makes taxonomy consistent with the phylogenomic hypothesis presented in Figure 27.2. However, it is striking that the genetic diversity of the *R. palustris* clade is larger than that of the neighbor *Bradyrhizobium* spp. clade, as judged from the (patristic) distances measured on the tree. This suggests that *R. palustris* represents a species assemblage, rather than a proper species. A combination of multilocus sequence-based phylogenetic and population genetic analyses should illuminate this issue (Vinuesa, 2010; Vinuesa et al., 2005).

27.3.3 Pangenome Phylogeny of the *Rhizobiales* Based on the Pangenome Matrix

As discussed above, and shown in Figure 27.1e and f, the core-genome represents only a minor fraction of the whole genome of an individual strain or of the pangenome of a certain species or higher taxon, as the order *Rhizobiales* analyzed herein. It is well known that adaptive evolution in prokaryotes is largely determined by gain and loss of genes (Doolittle, 2005; Gogarten and Townsend, 2005; Koonin and Wolf, 2008; Ochman et al., 2000), which have drastically shaped genome evolution in *Alphaproteobacteria* (Boussau et al., 2004). It would therefore be instructive to get an estimate of phylogeny based on the pangenome matrix, that is, the matrix of presence–absence data for each gene family. The rationale here is that the more similar two strains are in genome composition, the higher the likelihood that they will share the same or a similar niche (Gogarten and Townsend, 2005; Lukjancenko et al., 2010; Snel et al., 1999; Snipen and Ussery, 2010). The *parse_pangenome_matrix.pl* script will use the pangenome_matrix.tab file generated by *compare_clusters.pl* to compute a pangenome tree using the parsimony criterion, as implemented in pars from the PHYLIP package (Felsenstein, 2004). Figure 27.3 shows the pangenome parsimony tree computed from the consensus pangenome matrix for 68 *Rhizobiales* from the 14,297 consensus clusters generated by the COG and OMCL algorithms. Relationships within generic lineages remain largely consistent with those revealed by the core-genome phylogeny. However, some changes are apparent. For example, the *Brucella/Ochrobactrum* clade (containing facultative intracellular pathogens of humans and animals) is now sister to the *Mesorhizobium/Agrobacterium-Rhizobium/Sinorhizobium* clade, which groups the plant symbionts or pathogens with larger genomes. The strong genome reduction experienced by the *Bartonella* clade causes it to cluster in a basal position to the above-mentioned groups. Another interesting difference is that on the pangenome phylogeny *Bradyrhizobium* is sister to *Rhodopseudomonas*, while *Nitrobacter* clusters with *Oligotropha*. The former genera contain metabolically highly versatile organisms, exhibiting some of the largest genomes in the *Alphaproteobacteria*.

The *parse_pangenome_matrix.pl* script can also be used to identify lineage-specific gene families or family expansions. Owing to space constraints, such analyses are not presented herein. The reader is referred to the original publication describing GET_HOMOLOGUES for an example of how to use the pangenome phylogeny to guide the definition of pairs of groups to identify lineage-specific gene families or family expansions (Contreras-Moreira and Vinuesa, 2013).

27.3.4 Analyzing Large Datasets Using the Berkeley-DB and/or the Heuristic BDBH Algorithm Implementation

An important feature of the *get_homologues.pl* script is that it can call the standard Berkeley DB (BDB) database via the core DB_File Perl module. BDB is a software library that provides a high performance embedded database for key/value data. *get_homologues.pl* can be called with the −s flag to store sequence data temporarily in this database to reduce the memory footprint at the expense of larger CPU time. This allows the analysis of dozens of bacterial genomes on modest desktop computers with <10 GB RAM. Figure 27.4 shows a graph of memory usage by get_homologues.pl when called with BDB and without (default).

To speed up the process of analyzing hundreds of genomes, the script can be called with the −b option, which will run a heuristic version of the BDBH algorithm that runs a minimum number of BLAST searches. As explained in detail in the manual provided with the software package,

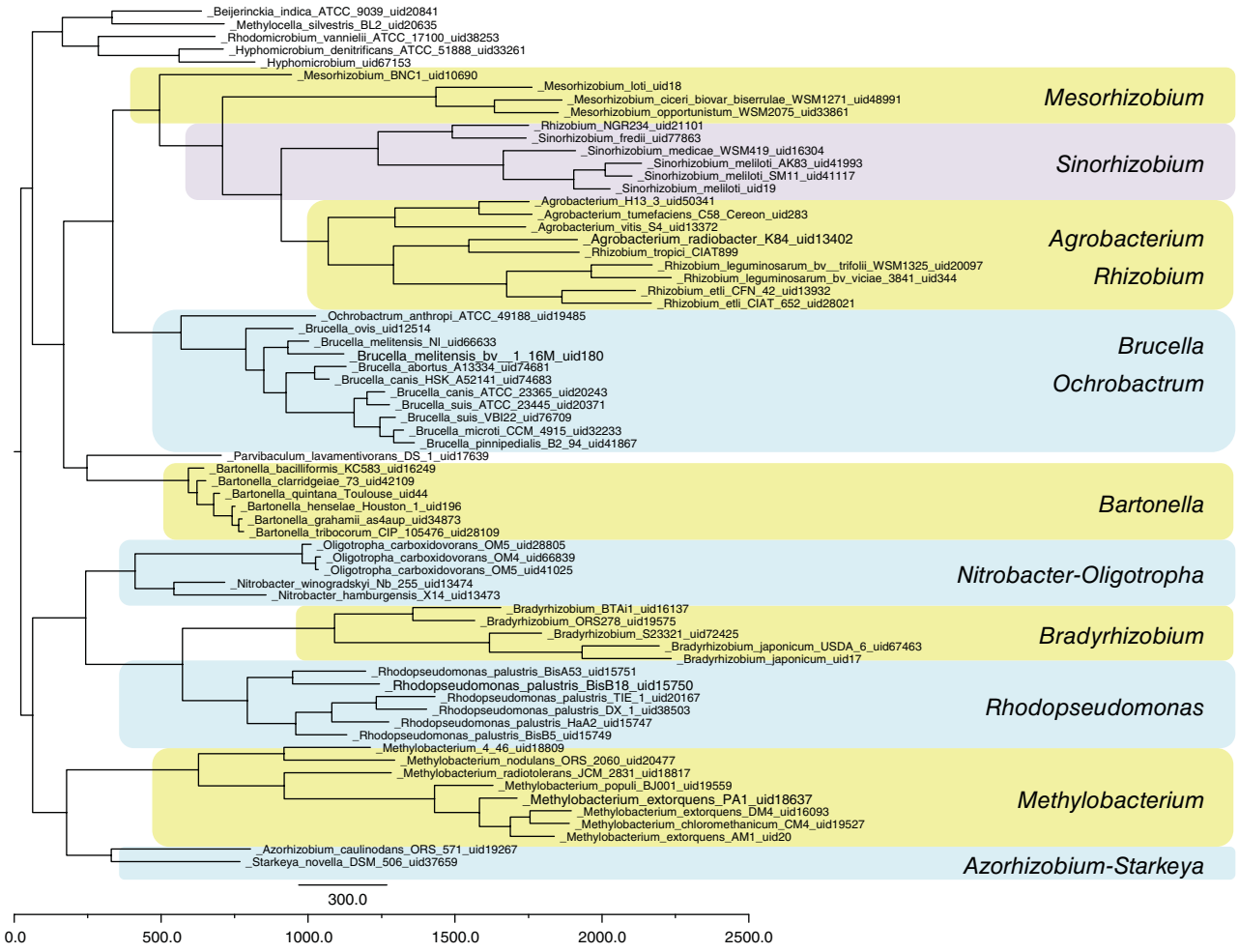

Figure 27.3 Pangenome tree based on the consensus pangenomic matrix (presence–absence matrix) containing 14,297 gene families derived from the intersection of COGtriangles and OrthoMLC clusters. The tree was recovered under the parsimony criterion. The scale indicates the number of gene clusters.

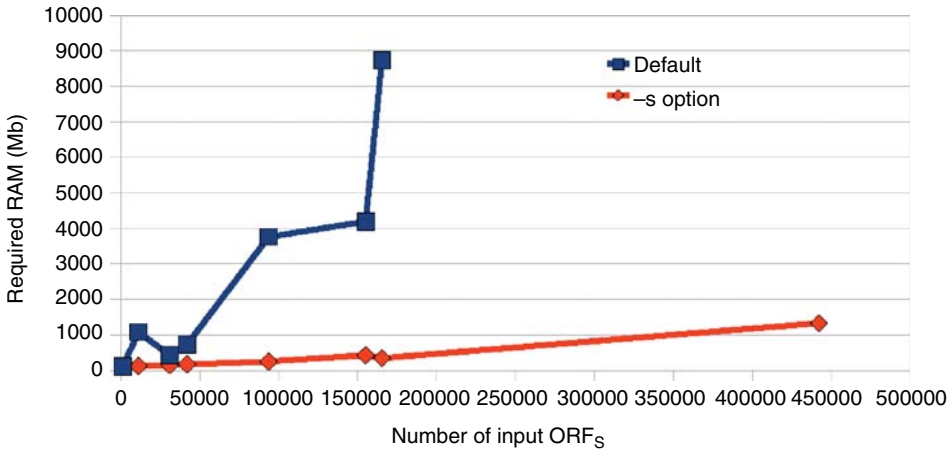

Figure 27.4 RAM requirement in Mb of the main script *get_homologues.pl* as a function of the number of genes to be analyzed when run in default mode or with the −s option enabled, which calls Berkeley DB to temporarily store data to disk to free RAM memory.

this option is probably only useful for the calculation of conservative core genomes.

27.4 CONCLUSIONS AND PERSPECTIVES

As briefly demonstrated in this work, the GET_HOMOLOGUES software package allows an easy, flexible, and thorough analysis of microbial pangenomes, computing very robust consensus core- and pangenomes and allowing for basic comparative genomics studies. The software is designed to take advantage of modern multiprocessor and multicore architectures, as well as of computer clusters to parallelize time-consuming hmmscan and BLAST jobs. The optional use of BDB to reduce the memory footprint permits the analysis of hundreds of genomes with moderate RAM resources. Altogether, these features allow non-bioinformaticians to easily perform sophisticated pangenome and basic comparative genomics analyses on large collections of complete or draft genomes, similar to those recently reported for different *Rhizobiales* (Sugawara et al., 2013; Tian et al., 2012). The robust consensus core genomes computed by the software permits refined phylogenomic analyses to be performed, as shown herein. The user of GET_HOMOLOGUES can upload selected gene families to our primers4clades server to select degenerate polymerase chain reaction (PCR) primers based on our extended CODEHOP algorithm for diversity and gene-based metagenomic studies (Contreras-Moreira et al., 2009; Sachman-Ruiz et al., 2011). We are currently developing computational pipelines that use the consensus orthologous gene families obtained by GET_HOMOLOGUES with the degenerate primer design capabilities of the primers4clades algorithm to develop highly informative markers for molecular ecology and systematics of rhizobia and other bacteria (Vinuesa, 2011).

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Section 6

Physiology and Metabolism of Nitrogen Fixing Organisms

Chapter 28

Metabolism of Photosynthetic Bradyrhizobia during Root and Stem Symbiosis with *Aeschynomene* Legumes

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28.1 INTRODUCTION

Rhizobia define a polyphyletic group of soil bacteria able to fix nitrogen and to interact in a symbiotic way with some plants of the legume family (Sprent, 2008). This interaction starts in the soil where plants and rhizobia exchange diffusible signal molecules, essentially plant flavonoid molecules (see Chapter 50) and lipochitooligosaccharides referred to as *Nod factors* (for nodulation factor) secreted by the bacteria (see Chapter 51). Once the reciprocal recognition is achieved, infection occurs and the plant develops nodules, which are new root organs hosting the bacteria. In the nodules, the symbiotic association is based on a nutrient exchange: the bacteria provide assimilable nitrogen to the plant under the form of ammonium, and in return, the plant supplies energy and carbon source to the bacteria (White et al., 2007). In the nodules, the bacteroid's metabolism is highly simplified compared to the free-living state, and seems to be totally reoriented in order to perform an efficient nitrogen fixation. The reduction of dinitrogen (N_2) to NH_3, which arises in rhizobia only during symbiosis, is an extremely energy-consuming process requiring both reducing equivalent and adenosine triphosphate (ATP) (White et al., 2007). These requirements are provided by the plant in the form of dicarboxylic acids. The bacteria assimilate this carbon source via the tricarboxylic acid (TCA) cycle and the malate dehydrogenase routes (White et al., 2007). Part of the resulting reducing equivalents is then converted into ATP through the microaerobic respiratory chains. These statements are likely true for all rhizobia–legume interactions. Nevertheless, variations occur around this theme.

We have been interested in a rhizobium/legume symbiosis established between photosynthetic bradyrhizobia and tropical aquatic legumes of the *Aeschynomene* genus. Five traits make this interaction atypical. First, bacteria involved in this interaction are photosynthetic. This feature

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is very rare among rhizobia and is only shared by some *Methylobacterium* strains nodulating *Lotonis* species (Norris, 1958; Jaftha et al., 2002). Second, plants of this genus are able to form symbiosis on both stem and root system (Heagerup, 1928; Alazard, 1985) (see Chapter 45 of this book). Third, these bacteria are able to reduce dinitrogen in their free-living state (Alazard, 1990). Fourth, they are also found as endophytic organisms associated with the wild rice [*Oryza brevigulata*] that grows spontaneously in natural wetland sites in close association with *Aeschynomene* species (Chaintreuil et al., 2000). The fifth and last exceptional aspect of these rhizobia is that the genomes of two strains [ORS278 and BTAi1] do not contain the canonical *nodABC* genes required to produce Nod factors (Giraud et al., 2007). Strikingly, despite this lack, their ability to form symbiotic association with *Aeschynomene* legume plants is not compromised. The wide range of environments that they are able to colonize – soil and wetland environments rhizosphere, shoot and root surfaces, cortical tissues, stem and root nodules – implies that they have evolved metabolic adaptations to ensure survival under these different conditions. The aim of this chapter is to review the current knowledge on photosynthetic *Bradyrhizobium* metabolism during stem and root nodule symbioses. More general aspects of rhizobial bacteroid metabolism have been recently reviewed by Terpolilli and coworkers (2012).

28.2 PHOTOSYNTHESIS AND SYMBIOSIS

Unlike cyanobacteria, the stem-nodulating bradyrhizobial symbionts of *Aeschynomene* are anoxygenic photosynthetic bacteria; this means that photosynthesis and nitrogen fixation can occur simultaneously in the same bacterial cell without risk of damage of the nitrogenase by oxygen released by the photosystem (PS). The PS of the bradyrhizobia and purple bacteria are very close but differ as the bradyrhizobial PS is not functional under anaerobic conditions (Giraud et al., 2000). It is composed of a photochemical reaction center and light-harvesting (LH) complexes. The genes necessary for the formation of the PS are clustered in a 45-kb region that is syntenic to the photosynthesis gene cluster of *Rhodopseudomonas palustris*, a bacteria phylogenetically close to *Bradyrhizobium* (Giraud and Fleischman, 2004). This cluster is illustrated in Figure 28.1a; it includes genes involved in photopigment synthesis (bacteriochlorophyll (*bch*) and carotenoids (*crt*)), genes encoding the LH polypeptides (*pufBA*), genes encoding the reaction center subunits (*pufLM*), and various regulators (*ppsR1, ppsR2, BrBphP* and *aerR*); see (Elsen et al., 2005 and Fig. 28.1a). Several independent studies indicate that this PS is functional during stem nodule symbiosis and contributes to the nitrogen fixation efficiency: (i) bacteroids isolated from stem nodules express a PS (Giraud et al., 2000), (ii) illumination of stem nodules with near-infrared light that is absorbed by bacteriochlorophyll but not chlorophyll enhances nitrogen fixation (Evans et al., 1990), and (iii) the growth of the plants inoculated with a photosynthetic mutant is 50% less than that of plants inoculated with the wild-type strain (Giraud et al., 2000); see Fig. 28.2.

It is clear that the plant benefits from the advantages conferred by this bacterial photosynthetic activity. Indeed, the energy collected by the bacterial PS can be used for stimulating nitrogen fixation and represents energy saving for the plant. This functional advantage could explain why essentially all the bradyrhizobia isolated from stem-nodulating *Aeschynomene* species have been found to be photosynthetic (Miché et al., 2010). On the other hand, this raises the question why the other stem-nodulating legume species, such as *Sesbania rostrata,* are interacting with non-photosynthetic symbiotic bacteria.

Regulation of PS formation in *Bradyrhizobium* is quite unusual in comparison to what is known in purple bacteria, since PS formation requires a low oxygen tension but also specific light conditions (Giraud and Fleischman, 2004). The fine control of PS formation by light requires a light sensor – the bacteriophytochrome (BrBphP; see Fig. 28.1a) that switches between two photo-interconvertible forms (Pr and Pfr) absorbing red and far-red light respectively

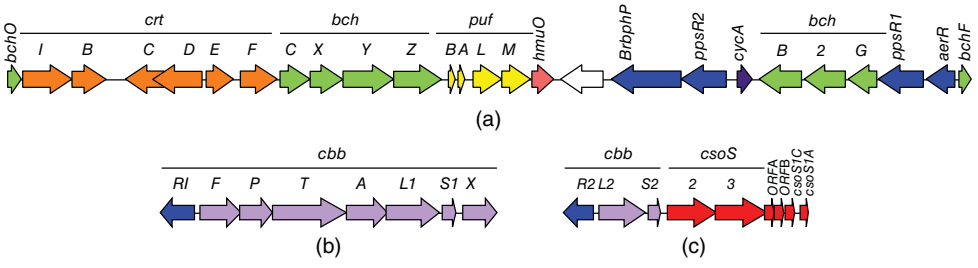


Figure 28.1 Organization of the photosynthesis gene cluster (a), the Calvin cycle gene cluster (b), and the carboxyzome gene cluster including the RuBisCO2 encoding genes (c). In (a) genes involved in carotenoid, bacteriochlorophyl synthesis are represented in orange and green, respectively; the *puf* genes encoding light harvesting polypeptides and reaction center subunits are colored in yellow. In (b,c) genes encoding enzymes of the CBB cycle and carboxyzome are colored in purple and red, respectively. In (a–c) genes encoding the regulator are represented in blue.

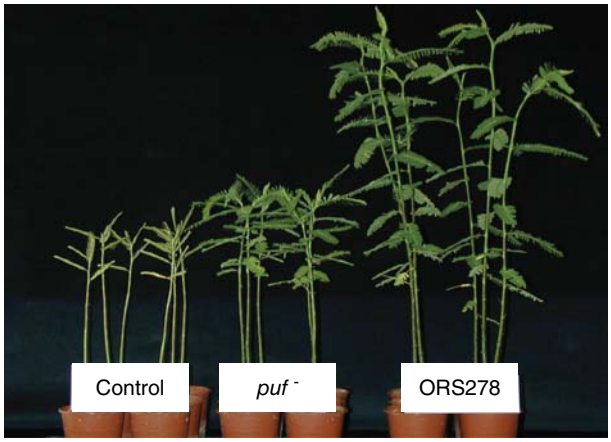
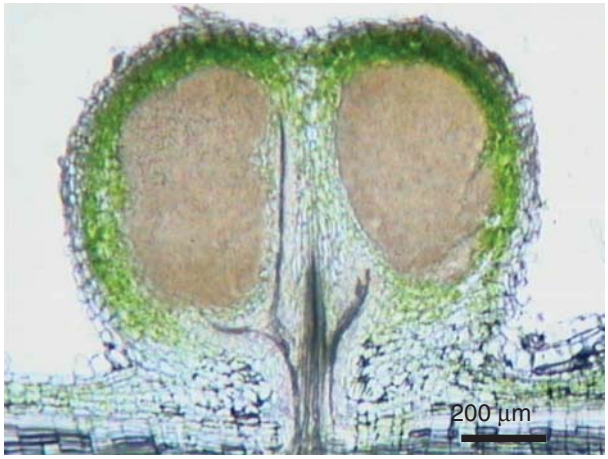


Figure 28.2 Bacterial photosynthesis is required for efficient symbiosis between *Aeschynomene sensitiva* and *Bradyrhizobium sp*. Strain ORS278. *A. sensitiva* plants were stem inoculated with WT or Δ*pufLM* ORS278 or not inoculated (control) and imaged 5 weeks after inoculation.

(Giraud et al., 2002). When BrBpHP is in its active Pr form, it allows the expression of the PS genes by antagonizing the transcriptional repressor PpsR2 (see Fig. 28.1a). At the same time, PpsR1 (see Fig. 28.1a), another transcriptional factor of the PpsR family, binds to the same promoter regions and activates the expression of the PS genes depending on the oxygen tension level. In other words, PS formation in *Bradyrhizobium* is under the dual control of two PpsR transcriptional factors that play antagonist roles and the actions of which are modulated by oxygen tension and light (Jaubert et al., 2004; Elsen et al., 2005). This atypical regulation of PS formation seems to be perfectly adapted to promote PS synthesis during stem symbiosis. Indeed, on the one hand, leghemoglobin maintains an oxygen tension that is compatible with the nitrogenase functioning and also with the activation of PS synthesis genes. On the other hand, the light perceived by the bacteria is first filtered through the nodule cortex (see Fig. 28.3), which contains chloroplasts preferentially absorbing blue and red light while transmitting far-red light. The enrichment in far-red light in the nodule tissue will then trigger PS synthesis thanks to the switch of the bacteriophytochrome into its active Pr form.

On the contrary, in the root nodules, i.e., when the root system is developed in the dark, no PS is formed. This means that light control by BrBpHP allows the bacteria to switch from dark heterotrophy during root symbiosis to the more energetically favorable photoheterotrophy during stem symbiosis. Interestingly, when the root system is exposed to light, for example, when plants are cultivated in Gibson tubes, both chloroplast formation in the root nodule cortex and bacterial PS formation occur (Fig. 28.3). This clearly indicates that light regulates plant energetic metabolism also through the induction of chloroplast differentiation in the nodule cortex covering the infected zone (see Fig. 28.3).

(a)



(b)

Figure 28.3 *Aeschynomene* root nodules exposed to light develop chloroplasts in the nodule cortex surrounding the infected zone. *Aeschynomene indica* root system (a) and transversal section of an *A. indica* root nodule (b).

To our knowledge, nodules containing chloroplast have only been described in *S. rostrata* (Dreyfus and Dommergues, 1981; James et al., 1996). We can easily deduce that the plant benefits from producing photosynthetates in the vicinity of the symbiotic cells, the nodule thus becoming an energetically independent functional unit. Furthermore, it is also worth highlighting that the photosynthetic apparatus of plants and bacteria that contain chlorophyll and bacteriochlorophyll, respectively, are not in competition for light because these photopigments absorb at different wavelengths. Their concomitant actions optimize light collection over a broader spectrum.

Beside the control of PS synthesis, one can expect that light controls other aspects of bacteroid metabolism. Indeed, it has been shown in *R. palustris* that the bacteriophytochrome RpBphP1, the homolog of BrBphP1, activates PS formation and also downregulates respiratory activity by repressing the synthesis of the alpha-ketoglutarate dehydrogenase complex, a central enzyme of the TCA cycle

(Kojadinovic et al., 2008). Indeed, the growth defect also observed for ORS278 strain when the cells are exposed to an inactinic far-red (770 nm) light (Verméglio André, personal communication) suggests that this dual control of both photosynthesis and TCA cycle should also occur *in nodulo* to enhance the most favorable bioenergetic process upon light exposure.

In nature, stem nodules are subjected to the alternation of day and night, meaning that the energetic metabolism of bacteroids is likely to drastically fluctuate over a 24-h period. Cyanobacteria possess an internal biological clock composed of three proteins – KaiA, KaiB, and KaiC – that enables them to anticipate and adjust their metabolism to the predictable diurnal cycle (Dong and Golden, 2008). Interestingly, sequence genome analysis reveals that photosynthetic bradyrhizobia possess two *kaiBC* operons, such genes not being found in non-photosynthetic rhizobia. It would then be particularly interesting to investigate whether these *kaiBC* genes are functional and allow a circadian control of certain aspects of bacteroid metabolism during stem symbiosis.

28.3 THE CALVIN CYCLE AND SYMBIOSIS

The Calvin–Benson–Bassham cycle (CBB cycle) is the major metabolic pathway responsible for biomass production at the global level. In algae, plants, and some prokaryotes, it allows CO_2 fixation and carbon incorporation in the biomass. The key enzyme of the CBB cycle is ribulose-bis-phosphate carboxylase oxygenase (RuBisCO). This enzyme converts one CO_2 and one ribulose-bis-phosphate molecule into two molecules of 3-phosphoglycerate. The rest of the metabolic pathway achieves the regeneration of the CO_2 acceptor. In addition to RuBisCO, only one other enzyme, the ribulose monophosphate kinase, is specific for the CBB cycle. The other reactions/enzymes are shared with sugar-related anabolic or catabolic pathways such as pentose phosphate, glycolysis, or neoglucogenesis. The CBB cycle is often associated with photosynthesis; however, one should keep in mind that many microorganisms do not necessarily combine photosynthetic ability with CO_2 fixation. For instance, the CBB cycle is functional in the photosynthetic *Bradyrhizobium* sp. BTAi1 (Hungria et al., 1993), as well as in the non-photosynthetic *Sinorhizobium meliloti* 1021 strain (Pickering and Oresnik, 2008) and *Bradyrhizobium japonicum* strain SR (Maier, 1981).

In photosynthetic bradyrhizobia, the genome of which has been sequenced, most genes involved in the CBB cycle are clustered in an operon-like structure and are located in close vicinity of the photosynthesis gene cluster. This cluster contains the so-called *cbb* genes, including *cbbL1* and *cbbS1,* encoding the large and the small subunits of the RuBisCO, respectively (see Fig. 28.1b). Interestingly, another gene set encoding a RuBisCO enzyme, named RuBisCO2, is present in the ORS278 strain genome (see Fig. 28.1c). This second copy is included in a cluster that also encodes carboxysome components. Carboxysomes are proteic microcompartments responsible for CO_2 concentration around RuBisCO (Price et al., 2008). This second gene cluster has likely been recently acquired as it harbors several typical features of lateral gene transfer (Giraud et al., 2007).

In this bacterium, only the RuBisCO encoded by genes located in the vicinity of the photosynthetic gene cluster is required to form an effective symbiosis with *Aeschynomene indica* (Gourion et al., 2011). A *cbbL1* mutant is not altered in its capacity to infect plants and to trigger nodule formation, but in contrast to the wild type (WT), plants infected by this mutant display a reduced nitrogen fixation activity. They form more nodules that are a mixture of reddish (functional) and yellowish (non-functional) organs. Expression studies using a promoter/reporter fusion confirm that *cbbL1* is constitutively expressed during the symbiotic process and the corresponding protein has been detected within nodules. In contrast, neither promoter activity nor protein could be detected for *cbbL2* in the same condition, i.e., in root nodules developed in the dark.

CO_2 fixation via the CBB cycle requires a substantial amount of ATP and reducing equivalents. In nodules developed in the dark, bacteria cannot use their photosynthetic ability to produce the energy necessary to run the CBB cycle. Instead, it is very likely that they rely on the plant partner for their energy supply. It is thus difficult to imagine that bacteria use the CBB cycle as a carbon supply machinery under symbiotic condition in darkness. Indeed, it would be futile for the bacteria to oxidize plant-derived organic carbon to generate energy dedicated to reduce CO_2 for forming organic carbon. The CBB cycle is used by some microorganisms as an electron sink, with CO_2 then acting as an electron acceptor (Joshi and Tabita, 1996; McKinlay and Harwood, 2010). Such a strategy would only make sense in environments where CO_2 is available and in which bacteria produce an excessive amount of reducing equivalents compared to their respiratory chain capacity. In nodules, bacteria are likely to encounter such conditions. Indeed, the respiratory chain capacity of the bacteria is reduced due to a low oxygen concentration and the TCA cycle produces a substantial amount of reducing equivalents and CO_2. If we consider the "electron sink hypothesis," the need of CO_2 as an electron acceptor should be only transient, mainly during the first steps of establishment of the symbiotic process before bacteroid differentiation, because when the bacteroids become functional, nitrogen fixation consumes a consequent amount of reducing equivalent and could take over the cofactor recycling. We could also imagine that other bacteria developed different strategies to solve the redox imbalance problem. Other metabolic pathways such as poly-ß-hydroxybutyrate

(PHB) synthesis could have an effect similar to CBB cycle activity (Terpolilli et al., 2012).

Alternative roles of RuBisCO in the symbiotic process cannot be excluded. Among them are the production of essential intermediary metabolite(s) by the CBB cycle or the photorespiration or the reduction of O_2 concentration by the RuBisCO Oxygenase activity to prevent nitrogenase damage.

Until now, the importance of RuBisCO for the symbiotic process has only been described in *Bradyrhizobium* sp ORS278. Nevertheless, the facts that (i) the phenotype does not involve the photosynthetic capacity of this bacterium, (ii) the *cbb* genes are widespread among rhizobia and, as mentioned earlier, (iii) the CBB cycle is functional in other rhizobia raise the question of the potential role of the CBB cycle in other symbiotic systems.

28.4 ADDITIONAL ASPECTS OF BACTEROID CENTRAL METABOLISM

Recent insights into the bacteroid metabolism during root symbiosis come from the screening of a large *Tn5* insertional library (>15,000 mutants) of the *Bradyrhizobium* ORS278 strain for their inability to fix nitrogen (Bonaldi et al., 2010).

From this genome-wide mutational analysis, major metabolic routes employed by *photosynthetic* bradyrhizobia during root symbiosis with *Aeschynomene* spp. have been revealed. These include the TCA cycle, glycolysis/neoglucogenesis pathways, PHB and glycogen biosynthesis/degradation, and as discussed above, the CBB cycle. This indicates that the central carbon metabolism of photosynthetic bradyrhizobia during root nodule symbiosis should not be drastically different from that of other rhizobia. All the genes encoding enzymes of the central metabolism required for efficient nitrogen fixation are listed in Table 28.1; for a complete list of genes involved in the symbiotic process refer to Bonaldi et al. (2010).

Below we highlight some original aspects or complementary information that could be deduced from this genetic screen:

- The TCA cycle plays a central role in the metabolism of rhizobium during symbiosis because it allows the metabolization of the dicarboxylic acids that represent the main carbon source provided by the host plant. The identification of Fix^- mutants in genes encoding several key enzymes of the TCA cycle indicates that the functioning of this cycle is indispensable for an efficient symbiosis between photosynthetic *bradyrhizobia* and *Aeschynomene*. Interestingly, these mutants displayed the same Fix^- phenotype when inoculated on the stem, suggesting that despite their ability to develop a photosynthetic apparatus and to fix CO_2, photosynthetic bradyrhizobia require to be fed by the plants with an appropriate carbon source.
- Operation of the TCA cycle requires anaplerotic synthesis of acetyl-CoA. It has been proposed that acetyl-CoA is mainly generated in bacteroids from the combined activities of malic enzyme and pyruvate dehydrogenase (PDH) complexes. An ORS278 mutant strain affected in BRADO3367, which encodes an NAD^+ dependant malic enzyme (annotated DME), has been identified. Interestingly this mutant is only slightly affected in its symbiotic efficiency, since it retained 86% of the WT nitrogenase activity. The Tn*5* insertion site is located at the beginning of the *dme* gene excluding the possibility that the mutated protein maintained a residual malic enzyme activity. As described in other rhizobia, strain ORS278 possesses an additional malic enzyme (BRADO5322), which is $NADP^+$ dependent (annotated TME). We therefore speculate that TME is functional during symbiosis and partially rescues the DME mutation. Because in *S. meliloti* TME fails to replace DME in N_2-fixing bacteroids (Mitsch et al., 2007), TME enzymes would play different physiological roles in both organisms.
- The ORS278 genome displays two *lpd* genes (BRADO0409 and BRADO4083, 43% identical) that encode dihydrolipoamide dehydrogenase. LpD together with PdhA, PdhB, and PdhC forms the PDH complex. When interacting with SucA and SucB, it forms the α-ketoglutarate dehydrogenase complex. BRADO4083 is located just downstream of the *pdhABC* operon, and BRADO0409 is located just downstream of the *sucAB* operon. Interestingly, we identified mutants in both *lpd* genes and they displayed a very different symbiotic phenotype. Mutants in BRADO4083 were drastically impaired in their N_2 fixation capacity, whereas mutants in BRADO0409 retained 85% of the WT nitrogenase activity. These results suggest that the LpD activity of BRADO0409 cannot replace that of BRADO4083 for the formation of a functional PDH. In contrast, BRADO4083 could rescue the mutation in BRADO0409 resulting in a partially functional α-ketoglutarate dehydrogenase. Alternatively, the α-ketoglutarate dehydrogenase complex might not be essential for symbiosis. This possibility is supported by the lack of symbiosis defective mutants in our collection that are affected in aconitase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase activity. Similarly, in *B. japonicum,* these key enzymes of the TCA cycle are dispensable for nitrogen fixation (Thöny-Meyer and Künzler, 1996; Green and Emerich, 1997; Shah and Emerich, 2006), suggesting that both organisms do not require an intact TCA cycle to meet their energy needs.

Table 28.1 Genes involved in symbiotic nitrogen fixation in *Bradyrhizobium sp.* ORS278

	Relative Nitrogenase Activity
Nitrogenase biosynthesis	
nifH (BRADO5394)	80%
nifN (BRADO5436)	ND
nifE (BRADO5437)	ND
nifK (BRADO5438)	ND
Electron transfers	
put.electron transfer flavoprotein deshydrogenase (BRADO2020)	19%
cycK (BRADO2734)	27%
put. pyruvate ferredoxin/flavodoxin oxidoreductase (BRADO4317)	ND
etfA (BRADO6491)	24%
etfB (BRADO6492)	26%
Tricarboxylic acid cycle	
sucC (BRADO0404)	ND
sucD (BRADO0405)	ND
sdhA (BRADO234)	ND
furnA (BRADO5016)	4%
IpD (BRADO0409)	85%
Pyruvate and carbohydrates metabolism	
IpD (BRADO4083)	ND
pdhA (BRADO4087)	6%
pdhB (BRADO4086)	ND
dme (BRADO3367)	86%
ena (BRADO4094)	ND
gpmA (BRADO0157)	5%
ppdK (BRADO2034)	34%
put.amylomaltase (BRADO5815)	6%
cbbK (BRADO1122)	80%
cbbE (BRADO2842)	ND
cbbL1 (BRADO1659)	40%
Amino acids biosynthesis	
hisB (BRADO0212)	54%
aatA (BRADO5973)	50%
Nucleotides biosynthesis	
purK (BRADO1205)	58%
purC (BRADO2776)	32%
Cofactors biosynthesis	
nadB (BRADO2036)	59%
put.RibBA-like protein (BRADO2133)	4%
Transporters	
pstC (BRADO1675)	3%
pstB (BRADO1677)	ND
put.ABC transporter (BRADO3096)	22%
put.ABC transporter (BRADO4782)	5%
put.permease (BRADO5117)	ND
put.ABC transporter (BRADO6119)	4%
Others	
Int (BRADO0045)	69%
put.peptidylpropyl isomerase (BRADO0589)	17%
put.acylCoA dehydrogenase (BRADO2179)	6%
put.hydrolase (BRADO2938)	18%
put.sulfite reductase (BRADO3794)	47%
put.D-alanyl-D-alanine carboxypeptidase (BRADO4549)	10%

Table 28.1 (*Continued*)

	Relative Nitrogenase Activity
put.PhnMprotein (BRADO5368)	3%
mutL (BRADO6079)	26%
mutA (BRADO2682)	13%

The list is restricted to genes encoding enzymes of the central metabolism required for nitrogen fixation but not for nodule development. The nitrogenase activities of the corresponding mutant were determined using acetylene reduction assays and are expressed as percentages of the WT control. ND stands for not detected.

- To cope with the lack of sugars provided by the plants, it is known that Rhizobium uses the gluconeogenesis pathway to synthesize the pool of essential sugars that they need. Several ORS278 Fix- mutant strains affected in three key gluconeogenic enzymes were isolated: enolase (Eno-BRADO4094), phosphoglyceromutase (GpmA-BRADO0157), and pyruvate phosphate dikinase (PPDK-BRADO2034). This also highlights the essential role of this pathway during the *Bradyrhizobium–Aeschynomene* symbiosis. Interestingly, it has been observed that the *ppdK* mutant retained 34% of the WT nitrogenase activity, whereas the *eno* and *gpmA* mutants were completely Fix$^-$. The partial activity of the *ppdK* mutant most likely results from an alternative route for phosphoenol pyruvate (PEP) formation, involving the PEP carboxykinase (PCK). It is notable that *ppdK* mutants of *S. meliloti* are not altered in their symbiotic performances whereas *pcK* mutants are strongly altered in nitrogen fixation, indicating that PEP is mainly formed via PCK (Osterås et al., 1997). In contrast to *S. meliloti,* we can propose that the main gluconeogenesis pathway in ORS278 involves the combined action of PPDK and malic enzyme. This is probably also the case for *B. japonicum* since *ppdk* is highly induced in bacteroids (a > 30 fold change) in comparison with microaerobic cultures (Pessi et al., 2007).
- As previously stated, the accumulation of storage compounds such as PHB or glycogen could be a strategy used by the bacteria to re-equilibrate the redox state of the cofactor pools produced by the TCA cycle. Accumulation of PHB granules can clearly be seen in bacteroids isolated from mature root nodules of *A. indica* or *A. afraspera* (Bonaldi et al., 2011). In addition, ORS278 mutants in key enzymes of glycogen or PHB catabolism are drastically impaired in nitrogen fixation. This suggests that the accumulation of these storage compounds and/or their subsequent metabolization is important for an efficient symbiosis.

28.5 PLANT CONTROL OF BACTEROID MORPHOTYPES

During the Rhizobium–legume symbiosis, bacteria are ultimately internalized in their host cells and undergo further morphological and metabolic differentiation. One remarkable feature of the photosynthetic *Bradyrhizobium/Aeschynomene* symbiosis is the synchrony of this differentiation, which occurs between the fourth and the fifth day post-inoculation and is rapidly followed by the beginning of detectable nitrogen fixation activity (Bonaldi et al., 2011).

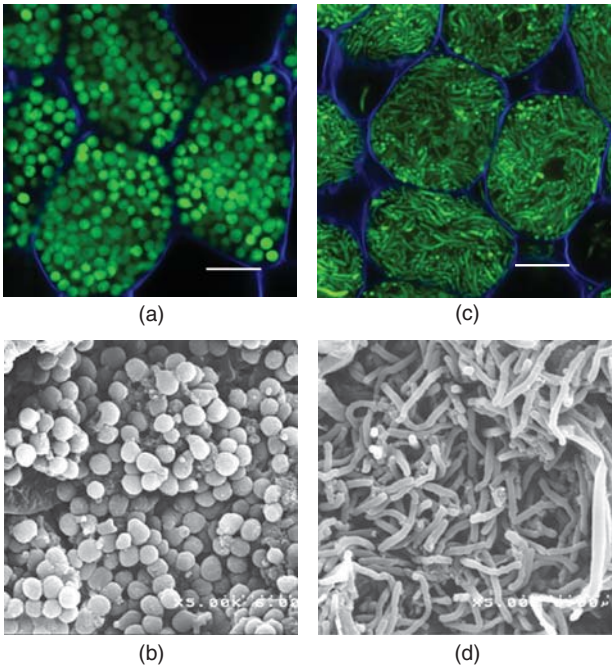


Figure 28.4 Various shapes of *Bradyrhizobium* sp. ORS285 strain bacteroids. ORS285 bacteroids are spherical in symbiotic cells of *Aeschynomene indica* (a,b) while elongated in *A. afraspera* (c,d). Images (a) and (c) were obtained with confocal microscope after calcofluor white staining of the cross sections of nodules and using a GFP-labeled *Bradyrhizobium* sp. ORS285 strain. Images (b) and (d) were obtained with scanning electron microscopy (b,d).

Interestingly, despite the taxonomical proximity between host plants, the morphology of bacteroids is drastically different depending on the host. Indeed, bacteroids are elongated in *A. afraspera* nodules, while they become spherical when in symbiosis with *A. indica* or *A. evenia* (Bonaldi et al., 2011) (see Fig. 28.4).

This is the first symbiotic system in which such closely related plants can trigger such drastically different shapes of the same bacteria. These observations indicate that in accordance with what has been previously described in the *Medicago–Sinorhizobium* system (Van de Velde et al., 2010); the bacteroid morphotype of photosynthetic bradyrhizobia is controlled by the plants. Mechanism(s) underlying this process remain(s) to be clarified, as well as the potential involvement of NCR-like or antimicrobial peptides (Van de Velde et al., 2010). This also raises the question of potential differences in symbiotic efficiency and bacteroid metabolism associated to these different bacteroid shapes.

28.6 CONCLUSIONS

To conclude, knowledge on photosynthetic *Bradyrhizobium*/*Aeschynomene* symbiotic systems is clearly far behind what is known about "classical" well-studied models. Nevertheless, several examples presented in this chapter highlight the utility to study such exotic models. Indeed, it has revealed atypical features of the symbiotic process not encountered in "classical models." Furthermore, it offers the possibility to investigate Rhizobium/legume interactions in relatively simple models, in which Nod factor signalization is not required. Now, the perspective of transferring symbiotic nitrogen fixation capacity to non-legume plants is returning in the discussions (Charpentier and Oldroyd, 2010). The possibility to bypass the requirements for Nod factor and for the associated signalization pathway is therefore of great interest (see also Chapter 108). Finally, we think these exotic models are of special interest to study differentiation aspects and associated variations thanks to the possibility to generate elongated or spherical bacteroids with the same bacterial strain in closely related plants.

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Chapter 29

A Plethora of Terminal Oxidases and Their Biogenesis Factors in *Bradyrhizobium japonicum*

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29.1 INTRODUCTION

The extant Alphaproteobacteria are phylogenetic relatives of mitochondria. In contrast to the latter, however, most species of this phylum possess cytoplasmic membrane-bound respiratory chains that are stunningly complex. There is diversity not only in the quinone-reducing enzymes but also in the routes electrons take from the quinols to their final destination, that is, the electron transport chain is often highly branched. A bacterium that reflects this complex situation in an almost unrivaled way is *Bradyrhizobium japonicum*. Under anoxic conditions, it is able to respire with nitrate as the terminal electron acceptor in a process called denitrification (Bedmar et al., 2005). The enzymes involved (nitrate, nitrite, nitric oxide, and nitrous oxide reductases) will not be the subjects of this article. Instead, the focus is on the respiratory oxygen reductases, generally called terminal oxidases, of which there are not less than eight in *B. japonicum* (Göttfert et al., 2004; Bühler et al., 2010). Six of them belong to the family of heme-copper oxygen reductases, and two are members of the copper-free cytochrome *bd* family. The level of information on the eight oxidases differs substantially, ranging from certain biochemical and molecular genetic details down to simple predictions derived from gene sequences.

Generation of reducing equivalents in *B. japonicum* occurs by oxidation of a huge array of known or potential carbon substrates (heterotrophy) and a few inorganic molecules (lithotrophy). While fermentation has never been reported, anaerobic nitrate respiration is possible (see earlier), but the main mode of consuming the reductants is clearly by electron transfer to oxygen as the terminal acceptor. The ubiquinone Q-10 is the major membrane-bound link in *B. japonicum* between the dehydrogenases and the terminal oxidases (Auling et al., 1988). Ubiquinols are oxidized either directly by quinol oxidases or via a chain that involves the ubiquinol:cytochrome *c* oxidoreductase (complex III; bc_1 complex), at least one *c*-type cytochrome, and finally the cytochrome *c* oxidases. Members of the alternative oxidase (AOX) family, originally thought to be restricted to mitochondria but recently found in prokaryotes as well (McDonald and Vanlerberghe, 2005), do not seem to exist in *B. japonicum*.

Incentives to study respiration in *B. japonicum* came from observations on the particular life styles of this bacterium. Being a soil bacterium, its aerobic energy metabolism is conceptually similar to that of many other aerobic prokaryotes in that habitat. The situation changes dramatically, however, when *B. japonicum* lives endosymbiotically within the cytoplasm of root nodule cells of

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its hosts (soybean, mung bean, cowpea, siratro). Herein, the bacteria – then called bacteroids – face an ambient concentration of free oxygen that is about four orders of magnitude lower than in the free-living state (~25 nM vs. 250 μM O_2); yet, they must be able to respire under these conditions (Hennecke, 1993). The search for a high-affinity, bacteroid-specific, and symbiotically essential oxidase has led to the discovery of the cbb_3-type cytochrome oxidase which, incidentally, turned out to be the prototype of a new subfamily within the heme-copper oxidase superfamily (Preisig et al., 1993, 1996a; Ducluzeau et al., 2008). Micro-oxia in root nodules is maintained because most of the oxygen is bound to the plant-derived leghemoglobin. Oxygen that dissociates from oxy-leghemoglobin is then respired by bacteroids with the help of cytochrome cbb_3, thus creating a nearly anoxic cytoplasm inside of the bacteria. This condition is perfectly suited for the function of nitrogenase.

This article not only addresses the diversity of terminal oxidases in *B. japonicum* but also the question how these enzymes are assembled in the membrane together with their cofactors. Proteins that act as chaperones or assembly factors play an important role in this context. Given that *B. japonicum* possesses eight terminal oxidases, this bacterium would seem to be a rather inconvenient choice for biogenesis studies. Fortunately, however, the expected complexity is partly alleviated by the fact that two of the eight oxidases, cytochromes aa_3 and cbb_3, predominate in aerobic culture and in symbiosis, respectively (Bühler et al., 2010). Mutations in the structural genes of cytochromes aa_3 and cbb_3 cause clearly discernible phenotypes, that is, defective cytochrome oxidase activity and symbiotic nitrogen fixation, respectively. These traits allow genetic studies on biogenesis proteins that are specifically destined to assemble either of the two oxidases.

29.2 THE DIVERSITY OF TERMINAL OXIDASES IN *B. japonicum*

Two illustrations may guide the reader through this section. Figure 29.1 shows the arrangement of genes for all of the eight terminal oxidases in *B. japonicum*. They form apparent operons in all cases. Only in one case (second row from top), the subunit structural genes are not contiguous but are interrupted by genes for cofactor synthesis and assembly (blr1172, blr1174). Genes whose products carry cofactors (diverse hemes and/or copper) are color-coded. Genes in the immediate neighborhood of these operons were omitted for clarity although they are occasionally relevant for the regulation, assembly, or *in vivo* functions of the associated oxidase.

Figure 29.2 illustrates the highly branched respiratory chain of *B. japonicum* and the placement of seven of the eight oxygen reductases at the end of the respective electron transport pathways. The heme-copper oxidase encoded by the bll4481-to-4479 genes (see top row in Fig. 29.1) could not be placed here for lack of evidence as to whether it is a quinol oxidase or a cytochrome oxidase. Further details on the contents of Figures 29.1 and 29.2 follow next, where many of the interpretations given were also influenced by available structural and biochemical data on related oxidases from other bacteria.

29.3 THE HEME-COPPER OXIDASES

The heme-Cu_B-containing subunit I is the common denominator in six of the eight terminal oxidases (Fig. 29.1), and five of them could be placed into the scheme of Figure 29.2 (highlighted in pink). The three cytochrome oxidases on the right (Fig. 29.2) receive the electrons from the bc_1 complex via *c*-type cytochromes. Which of the *c*-type cytochromes might be involved will be discussed later. Akin to many aerobic and photosynthetic bacteria, the bc_1 complex is composed of three subunits: the Rieske iron–sulfur protein, the cytochrome *b*, and cytochrome c_1. A peculiarity in *B. japonicum* is that the three proteins are encoded by a bicistronic operon (*fbcFH*) where *fbcF* codes for the Rieske protein and *fbcH* for a large precursor that must be proteolytically cleaved to release apocytochrome *b* and apocytochrome c_1 during the process of membrane insertion (Thöny-Meyer et al., 1989, 1991). *B. japonicum* mutants of *fbcFH* have a severe defect in root-nodule colonization and symbiotic nitrogen fixation, which had been taken previously as the first evidence for the existence of a bc_1-dependent electron transport chain – now known to lead to cytochrome cbb_3 – that is essential for the symbiotic life style (Fig. 29.2).

29.3.1 Cytochrome aa_3 and Its Apparent Homolog

The four-subunit cytochrome aa_3 encoded by *coxA* (subunit I), *coxB* (subunit II), *coxC* (subunit III), and *coxF* (probable subunit IV) (Fig. 29.1) is the most prominent terminal oxidase in aerobically grown *B. japonicum* cells (Bott et al., 1990; Gabel and Maier, 1990; Bühler et al., 2010). It accounts for practically all of the cytochrome oxidase activity in membranes of such cells when either reduced cytochrome *c* (from horse heart) or *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) is used as the electron donor. This means there is very little, if any, background activity in air-grown *B. japonicum coxA* or *coxB* mutants that might stem from other cytochrome oxidases. Curiously though, the mutants are hardly affected

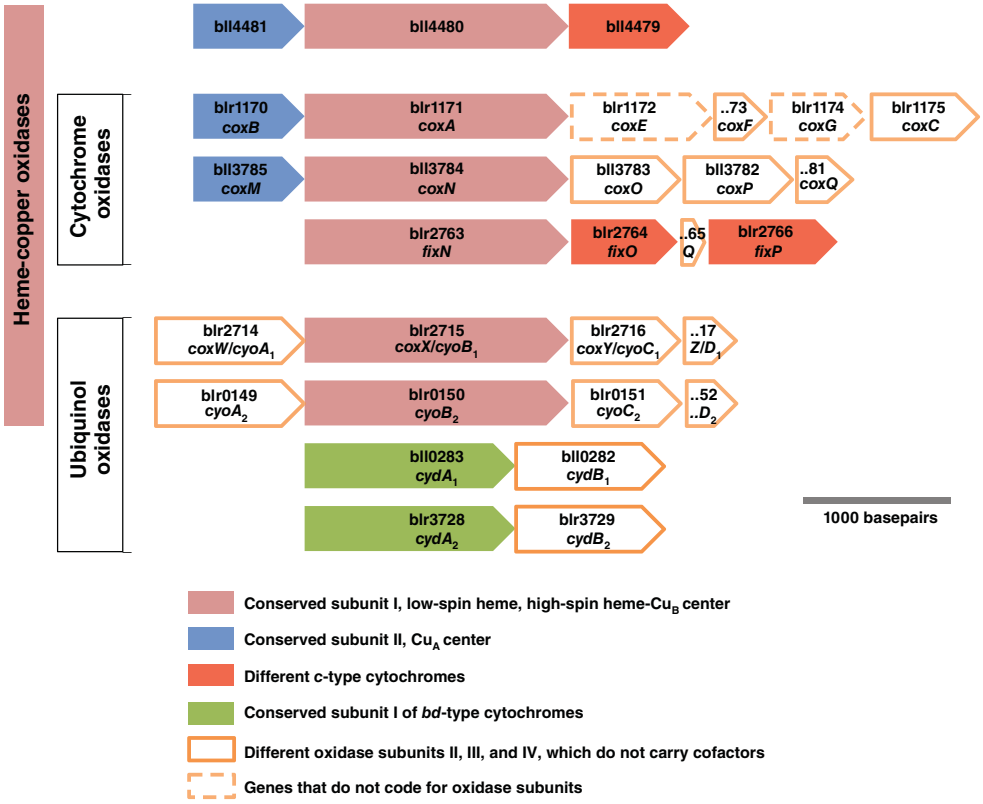

Figure 29.1 Gene clusters for the eight terminal oxidases in *B. japonicum*. The color code at the bottom is self-explanatory. The genes are marked with their numbers and, where available, with their names.

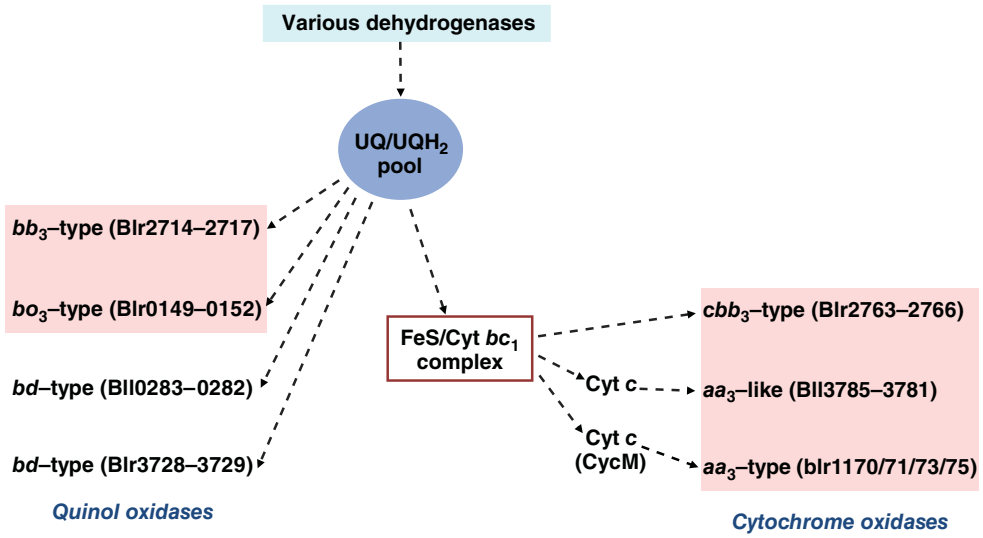

Figure 29.2 The branched respiratory chain of *B. japonicum*. The branches terminate either with quinol oxidases (left) or with cytochrome oxidases (right). Terminal oxidases that belong to the class of heme-copper oxidases are highlighted in pink color.

in aerobic growth that is interpreted to mean that respiration is sustained by the collective activity of ubiquinol oxidases expressed in such cells (Fig. 29.2, on the left). The mutants are also efficient in symbiotic nitrogen fixation, just like the wild type, which precludes a role of cytochrome aa_3 in symbiosis. In fact, expression of the *coxBACF* genes is downregulated in bacteroids of soybean root nodules (Gabel and Maier, 1993; Bühler et al., 2010).

```
Pd SU II     -H--34aa--CSELCGINHAYM-
Bj CoxB      -H--34aa--CSELCGKDHAFM-
Bj CoxM      -H--34aa--CAELCGAAHYQM-
Bj Bll4481   -H--34aa--CQEFCSFGHEGM-
Consensus    -H--34aa--CXEXCXXXHXXM-
```

Figure 29.3 Amino acid sequence motif of the copper A center in subunit II of heme-copper cytochrome oxidases. The alignment shows the respective sequence motifs from *P. denitrificans* (Pd) and *B. japonicum* (Bj). Compare Figure 29.1 for an affiliation of subunit-II proteins CoxB, CoxM, and Bll4481.

Extensive bioinformatic analyses of the *B. japonicum* cytochrome aa_3 subunits (sequence conservation, transmembrane helices, cofactor binding sites, domain topology) combined with few biochemical data strongly suggest that this oxidase and the redox centers in it have a structure similar to that of the *Paracoccus denitrificans* cytochrome aa_3 (Iwata et al., 1995). Accordingly, the low-spin heme-A iron and the metals of the binuclear heme A_3–Cu_B center in subunit I are liganded by two and four defined histidine residues, respectively. Subunit II carries a binuclear Cu–Cu center (Cu_A), which is complexed by six absolutely conserved amino acids as shown in Figure 29.3. Subunits III and IV do not carry cofactors.

There is evidence to suggest that cytochrome aa_3 forms a membrane-integral supercomplex together with the ubiquinol:cytochrome *c* oxidoreductase and the CycM protein, a membrane-anchored mono-heme cytochrome *c* that replaces soluble periplasmic cytochrome *c* in the transfer of electrons to the terminal oxidase (Fig. 29.2) (Bott et al., 1990, 1991).

The *B. japonicum* genome carries paralogs of the *coxBACF* genes, which had been named *coxMNOPQ* (Bott et al., 1992) (Figs. 29.1–29.3). A subunit III-like protein is encoded twice (*coxO* and *coxP*). The two genes might have been generated by duplication of a common ancestor during evolution. In all growth conditions tested, free-living and symbiotic, the *coxMNOPQ* genes are poorly expressed (Bühler et al., 2010), and mutations in them have so far not caused a phenotype other than wild-type (Bott et al., 1992). This has hampered any progress in the description of the biochemical and spectral properties of the encoded terminal oxidase, and its physiologic function remains enigmatic.

29.3.2 Cytochrome *cbb*$_3$

The common denominator in respiratory heme-copper oxidases is the membrane-integral subunit I, which is also present in the cbb_3-type oxidase (FixN or CcoN). Instead of heme A, however, it carries a low-spin heme B and a high-spin heme B-Cu_B center where reduction of O_2 to H_2O takes place (Buschmann et al., 2010). An exceptional property of cbb_3-type oxidases is subunit II (FixO/CcoO), which is a mono-heme cytochrome *c* instead of a Cu_A-containing subunit II. The FixP/CcoP subunit is usually a membrane-anchored di-heme cytochrome *c*, which is thought to relay electrons delivered by the cytochrome bc_1 complex (Fig. 29.2). The small, nonessential subunit FixQ/CcoQ might be involved in the assembly of the enzyme complex (Ekici et al., 2012a).

Research with the *B. japonicum* genes and enzyme has shown that (i) the *fixNOQP* operon is highly induced and essential in symbiosis (Preisig et al., 1993; Zufferey et al., 1996; Nellen-Anthamatten et al., 1998), (ii) the enzyme has a high affinity for molecular oxygen ($K_M = 7$ nM (Preisig et al., 1996a)), and (iii) the oxidase is capable of pumping protons over the membrane with an efficiency of 0.5 H^+/e^- (Arslan et al., 2000). All of these properties are compatible with the function of cytochrome cbb_3 as an oxygen reductase that sustains bacteroid respiration and energy conservation in the micro-oxic environment within root nodules. The recent report on the structure of a bacterial cytochrome cbb_3 has led to a possible explanation as to why the enzyme has a high affinity for oxygen (Buschmann et al., 2010). The authors noticed a distortion of heme in the heme B-Cu_B active site, which might facilitate O_2 access and binding.

29.3.3 In a Class of Its Own: bll4481–4479

The top row of Figure 29.1 shows genes whose deduced products clearly suggest the existence in *B. japonicum* of yet another heme-copper oxidase with a conserved heme-Cu_B-containing subunit I (bll4480) and a conserved Cu_A-containing subunit II (bll4481; see also Fig. 29.3). Evidence for associated subunits III and IV is missing; instead, the bll4481/4480 genes form an apparent operon together with the downstream gene bll4479, which codes for a di-heme *c*-type cytochrome (Fig. 29.1). This observation plus some other genes in the immediate vicinity of the operon (not shown) provide weak hints that this is a terminal oxygen reductase that receives the electrons from a pyrroloquinoline quinone (PQQ)-dependent dehydrogenase. The carbon substrate for such a specific oxidation pathway is completely unknown, and the analysis of knockout mutants of this gene region has so far not led to a discernible phenotype (Bühler et al., 2010). Likewise, it remains enigmatic as to whether the electron flow to this oxidase occurs via a quinol or via a reduced cytochrome *c*. Therefore, a placement in the scheme of Figure 29.2 was not possible.

29.3.4 Two Homologous Heme-Copper Ubiquinol Oxidases

An inspection of the genome has unveiled the existence of two homologous four-subunit oxidases in which the presence of a cofactor-free subunit II (i.e., without Cu_A) indicates that

they use ubiquinol rather than cytochrome *c* as the electron donor (Figs. 29.1 and 29.2). The only cofactor-containing protein is the heme-Cu_B-containing subunit I. Only one of the two quinol oxidases (blr2714–2717) has been characterized to some extent (Surpin et al., 1996; Surpin and Maier, 1999). The corresponding genes were previously named *coxWXYZ*, which is an inappropriate nomenclature because they do not code for a cytochrome oxidase. Based on spectral properties, the oxidase was classified as a bb_3-type oxygen reductase (Surpin et al., 1996). Interestingly, a *coxWXYZ* mutant had a marginally decreased symbiotic nitrogen-fixation activity (70% residual nitrogenase activity as compared with the wild type). Hence, apart from cytochrome cbb_3, the *coxWXYZ*-encoded ubiquinol oxidase might partially contribute to the respiratory activity of *B. japonicum* at some stage during nodule colonization (Surpin and Maier, 1999).

29.4 TWO CYTOCHROME *bd*-FAMILY MEMBERS

Completely unrelated to the heme-copper oxidases are the so-called *bd*-type quinol oxidases (Jünemann, 1997). The two-subunit *bd*-type oxygen reductases do not contain copper as cofactor, and their subunits do not share significant sequence similarity with subunits of the heme-copper oxidases. As the name says, they contain hemes B (two) and D (one) as cofactors, where the active site is generally composed of heme B_{595} and heme D in which the iron atoms of the two tetrapyrroles form a binuclear center (Borisov et al., 2011). The *B. japonicum* genome obviously encodes two homologs of this enzyme class (Fig. 29.1). Unfortunately, it is not known under which growth conditions these genes are expressed, which makes it difficult to attribute a specific function to them. Intriguingly, the bll0283-0282 genes are clustered together with genes for a malate degradation pathway (not shown). Yet, a mutant of bll0283 ($cydA_1$) was found to be unaffected both in free-living growth on malate and in symbiosis (Arslan, 2001). Heterologous expression of the *B. japonicum* $cydA_1B_1$ genes in an *Escherichia coli* strain that was depleted of its own three terminal oxidases revealed the spectrophotometric features of a *bd*-type oxidase, which underlined the functionality of *B. japonicum* $cydA_1B_1$ in *E. coli* (Arslan, 2001).

29.5 FACTORS INVOLVED IN THE BIOGENESIS OF CYTOCHROMES aa_3 AND cbb_3

The following paragraphs in this section describe all of the proteins that are relevant for the biogenesis of cytochrome oxidases, with special emphasis on those from *B. japonicum*. These are compiled in Table 29.1.

29.5.1 Surf1

Subunit I of aa_3-type heme-copper oxidases contains two molecules of heme A deeply buried within a hydrophobic pocket and functioning as cofactors for the O_2 reduction site. Heme A is synthesized from heme O by an enzyme termed heme A synthase and integrated into subunit I with the aid of a protein termed Surf1. Members of the Surf1 family of proteins are approximately 30-kDa inner mitochondrial membrane/periplasmic, membrane-anchored proteins exclusive to mitochondria/Alphaproteobacteria. They contain two transmembrane helices connected by a large loop facing the intermembrane space/periplasm (reviewed by (Hannappel et al., 2012)). Mutations in the *SURF1* gene are a frequent cause of Leigh's disease in children (Tiranti et al., 1998; Zhu et al., 1998; Darin et al., 2003), a usually fatal neurological disorder associated with severe cytochrome oxidase deficiency (Leigh, 1951). Surf1 proteins are not essential for cytochrome oxidase assembly but deletion of their genes substantially decreases activity of their associated oxidase (~60% loss in *P. denitrificans* (Bundschuh et al., 2008) and 80–90% in human tissues (Van Coster et al., 1991; Tiranti et al., 1999; Williams et al., 2004; Stiburek et al., 2005)) and causes a specific decrease in the heme A cofactor in aa_3-type cytochrome oxidase (Smith et al., 2005; Bundschuh et al., 2008). Surf1 copurifies with subunit I (Hannappel et al., 2012), and the transfer of heme A from heme A synthase to Surf1 has been demonstrated *in vitro* (Hannappel et al., 2011). The exact role of Surf1 in COX assembly is still unclear but it may facilitate heme A insertion into subunit I in one or more of the following ways: (i) protection of subunit I during heme A insertion; (ii) regulation of heme A synthase activity by controlling the release of heme A to subunit I; or (iii) insertion of heme A into subunit I. Given that free heme is toxic to the cell (Kumar and Bandyopadhyay, 2005; Anzaldi and Skaar, 2010), Surf1 may also be important to chaperone heme A and prevent it from being free within the membrane.

B. japonicum has two homologs of *surf1* within its genome. The first homolog, blr1177, is located at the end of the operon-encoding cytochrome *c* oxidase subunits I–III (*coxA*, *coxB*, *coxC*), *coxG*, and *coxE* (for heme O synthase) (see Fig. 29.1 for comparison). The second homolog, blr0153, is located downstream of the *cyo* operon-encoding subunits I–IV ($cyoA_2$-D_2) of a quinol oxidase (cf. Fig. 29.1). Other bacteria also have more than one homolog of *surf1* within their genome. In *P. denitrificans* there are two homologs of *surf1*; the first termed *surf1c* is located in an operon encoding the subunits of aa_3-type cytochrome oxidase, and the second termed *surf1q* is located in an operon encoding a ba_3-type quinol oxidase (Bundschuh et al., 2008). Deletion of either *surf1* homolog results in an exclusive loss of activity of its associated oxidase (Bundschuh et al., 2008). In *B. japonicum*, we have constructed

Table 29.1 Confirmed and putative cytochrome oxidase assembly factors in *B. japonicum*

Locus	Gene	Function	Target Oxidase	Mutant Available	Fix Phenotype	Reference
blr0467	*ccmA/cycA*	Synthesis of cyt *c*	cbb_3	Yes	Fix^-	Ramseier et al., 1991
blr0468	*ccmB/cycB*	Synthesis of cyt *c*	cbb_3	Yes	Fix^-	Ramseier et al., 1991
blr0469	*ccmC/cycC*	Synthesis of cyt *c*	cbb_3	Yes	Fix^+	Ramseier et al., 1991
bsr0470	*ccmD/cycX*	Synthesis of cyt *c*	cbb_3	Yes	Fix^-	Ramseier et al., 1991
blr0471	*ccmG/cycY*	Synthesis of cyt *c*	cbb_3	Yes	Fix^-	Fabianek et al., 1997
blr3125	*ccmI/cycH*	Synthesis of cyt *c*	cbb_3	Yes	Fix^-	Ritz et al., 1993
blr3126	*ccmE/cycJ*	Synthesis of cyt *c*	cbb_3	Yes	Fix^-	Ritz et al., 1995
blr3127	*ccmF/cycK*	Synthesis of cyt *c*	cbb_3	Yes	Fix^-	Ritz et al., 1995
blr3128	*ccmH/cycL*	Synthesis of cyt *c*	cbb_3	Yes	Fix^-	Ritz et al., 1995
blr1174	*coxG*	Copper chaperone	aa_3	Yes	Fix^+	Bühler et al., 2010
blr1131	*scoI*	Copper chaperone	aa_3/cbb_3	Yes	Fix^-	Bühler et al., 2010; Serventi et al., 2012
bll4880	*pcuC*	Copper chaperone	aa_3/cbb_3	Yes	Fix^-	Serventi et al., 2012
bll1380	*tlpA*	Reductant for ScoI/CoxB	aa_3	Yes	Fix^-	Loferer et al., 1993
blr1177	*surf1c/shb1*	Insertion of heme A	aa_3	Yes	Not tested	This work
bll4968	*cox15*	Heme A synthase	aa_3	Yes	Not tested	M. Bott, unpublished
blr1172	*coxE*	Heme O synthase	aa_3	No		Bühler et al., 2010
blr2767	*fixG*	Oxidation of Cu(I)?	cbb_3	Yes	Fix^-	Preisig et al., 1996b
blr2768	*fixH*	Unknown	cbb_3	Yes	Fix^-	Preisig et al., 1996b
blr2769	*fixI*	Copper transport?	cbb_3	Yes	Fix^-	Preisig et al., 1996b
bsr2770	*fixS*	Unknown	cbb_3	No		Preisig et al., 1996b

This table lists all genes confirmed or thought to be important for synthesis of the cytochrome oxidases in *B. japonicum*. This table also lists whether or not a mutant has been constructed, the Fix phenotype of the mutant, and relevant references.

deletion mutants of blr1177 and blr0153 and named them Δ*surf1c* and Δ*surf1q* in accordance with the aforementioned *P. denitrificans* homologs. As in *P. denitrificans*, deletion of *surf1c* (blr1177) but not deletion of blr0153 (*surf1q*) causes loss of activity of the aa_3-type cytochrome oxidase (Z. Youard and E. Rigozzi, unpublished results). We were unable to test the effect of the two mutations on activity of the cyo_2-encoded quinol oxidase because it is unknown at present under what conditions this oxidase is important for growth of *B. japonicum*.

29.5.2 Ccm System

As was described earlier, the symbiotically essential cbb_3-type cytochrome oxidase contains two subunits that are *c*-type cytochromes (FixO and FixP). Therefore, biogenesis factors involved in cytochrome *c* formation are symbiotically relevant as well. All *c*-type cytochromes contain at least one B-type heme that is linked covalently to the polypeptide by one to two thioether bonds. Attachment of the vinyls at positions 2 and 4 of the tetrapyrrole ring of heme to the thiols of the two cysteines (Cys_1 and Cys_2) of the heme-binding motif (C_1XXC_2H) of *c*-type apocytochromes (apocyt *c*) requires a series of membrane-associated biogenesis proteins. In Alphaproteobacteria and Gammaproteobacteria, archaea, mitochondria of plants, red algae, and in *Deinococcus* species, this pathway is referred to as the cytochrome *c* maturation (Ccm) system or System I (reviewed by (Sanders et al., 2010)).

The Ccm system can be separated into three operational modules (I–III) and consists of up to 10 membrane-bound proteins including CcmABCDEFGHI encoded by the *ccm* operon and CcdA (DsbD in some species). Module I consists of five components, CcmABCDE, of which the first four components (CcmABCD) are possibly involved in transporting and loading heme B onto CcmE (Schulz et al., 1999; Ren and Thöny-Meyer, 2001). CcmE seems to function as a chaperone to supply heme for module III and apocyt *c* (Mavridou et al., 2012). Module II consists of CcdA/DsbD, CcmG, and CcmH, and its proposed function is to prepare apocyt *c* for heme binding by reducing the cysteines of the heme-binding motifs to allow addition of heme B. It has been proposed that oxidation of these cysteines into intramolecular disulfide bonds occurs upon Sec-dependent translocation of apocyt *c* across the membrane via the extracytoplasmic DsbA-DsbB oxidative protein-folding pathway. CcdA/DsbD, CcmG, and CcmH are reducing agents that contain thioredoxin-like motifs (CXXC) for the reduction of disulfide bridges. Module III catalyses ligation of heme B to apocyt *c* to form holocytochrome *c* (holocyt *c*). The components of Module III appear to differ between species.

In *R. capsulatus*, for example, the activity of Module III has been attributed to CcmF, CcmH, and CcmI. The function of each component in Module III has not yet been determined, and various theories have been put forward to explain their importance for heme ligation. The Ccm system of *B. japonicum* has not been as well studied as the *E. coli* and *R. capsulatus* systems partly due to the difficulty of biochemical characterization within this species (lack of nonpolar *ccm* mutants, low abundance of Ccm proteins, and lack of a system for native overexpression). The *ccm* homologs in *B. japonicum* are organized into separated gene clusters, *cycABCXY* and *cycHJKL* (Ramseier et al., 1991; Ritz et al., 1993, 1995; Fabianek et al., 1997).

29.5.3 TlpA

Thioredoxins are ubiquitous proteins that share a common 3D architecture known as the thioredoxin fold, consisting of four α-helices and five β-sheets, and a highly conserved active-site sequence (Cys-Gly-Pro-Cys) (reviewed by Zeller and Klug (2006)). If soluble in the cytoplasm, these proteins form part of the thioredoxin system in which electrons are passed from NADPH to thioredoxin via thioredoxin reductase. Thioredoxin proteins have a low redox potential and are efficient reductants for disulfide bonds. Together with the glutaredoxins, thioredoxins help to maintain the intracellular reducing environment and play an important role in regulation of enzyme activity through redox-dependent signal transduction, a process termed the thiol switch (reviewed by Paget and Buttner (2003)).

In *B. japonicum* a thioredoxin-like protein, TlpA, was identified that shares approximately 31% amino acid sequence identity with various eukaryotic and prokaryotic thioredoxins and protein disulfide isomerases (Loferer et al., 1993). It possesses the same characteristic active-site sequence of all members of the thioredoxin family but is larger than other bacterial thioredoxins (23 kDa) and anchored to the cytoplasmic membrane with its active site-containing domain facing the periplasm. The *tlpA* mutant shows a pleiotropic phenotype with strongly decreased cytochrome *c* oxidase activity and defective development of nitrogen-fixing symbiosis with soybean compared to the wild type (Loferer et al., 1993).

Deletion of *tlpA* does not affect the expression or membrane insertion of the apoprotein of subunit I (CoxA), but it causes a decrease in the steady-state level of subunit I, suggesting that TlpA is involved in assembly/maturation or stabilization of cytochrome aa_3 (Loferer et al., 1993). Biochemical evidence from TlpA overexpressed in *E. coli* and purified without its membrane anchor ($TlpA_{sol}$) shows that it is a monomer in solution and catalytically active in reducing disulfides and oxidizing vicinal cysteine residues of heterologous proteins *in vitro* and can thus be classified as a protein thiol:disulfide oxidoreductase (Loferer and Hennecke, 1994). Further studies revealed that TlpA was a reductant, supporting the idea that the primary role *in vivo* of TlpA was to keep thiols of target proteins reduced (Loferer et al., 1995). One strong candidate for a TlpA target protein is ScoI. ScoI is proposed to function as a copper chaperone or disulfide reductase for delivery of copper into the active site of cytochrome aa_3. Regardless of the exact function of ScoI, its activity depends on the cysteines in its active site being in the reduced dithiol form for binding of copper (Bühler et al., 2010; Mohorko et al., 2012). In fact, TlpA was recently shown to reduce ScoI *in vitro* with a reaction rate constant ($9.4 \times 10^4\ M^{-1}\ s^{-1}$) that is typical for physiologically relevant disulfide exchange reactions, and it was determined that the redox potential of TlpA was more negative than that of ScoI ($E^{0'}$ −256 mV vs. −160 mV) (Mohorko et al., 2012). In the same study, a transient heterodisulfide bond was observed between Cys107 of the active site of TlpA and Cys78 of the copper-binding site of ScoI (Mohorko et al., 2012). This supports that ScoI is a biologically relevant target for TlpA, and that TlpA is important for recycling the active-site cysteines of ScoI prior to binding of copper.

A number of issues are currently being addressed, such as the identification of the system that is responsible for reducing/regeneration of the TlpA active-site cysteines, the identity of the target protein/s of TlpA responsible for the symbiosis-defective phenotype of the *tlpA* mutant, and finally, whether other proteins are also targeted by TlpA. Some of these issues will be reiterated after the discussion of ScoI function (see later). The X-ray structure of $TlpA_{sol}$ has been determined, and a number of residues have been identified that may play an important role in target recognition and activity of TlpA (Capitani et al., 2001).

29.5.4 ScoI

ScoI, also called SenC or PrrC, is a homolog of the mitochondrial copper chaperone Sco1 (reviewed by Robinson and Winge (2010)). Sco proteins (synthesis of cytochrome *c* oxidase) are cytoplasmic/inner mitochondrial membrane-anchored proteins with a large soluble domain that faces the periplasm/inner membrane space. Sco proteins bind to one copper ion via a CXXXC motif and a conserved histidine ligand. Sco proteins are widespread in eukaryotic and prokaryotic organisms, and some species possess more than one Sco homolog (Banci et al., 2011). Sco proteins have been implicated in the assembly of both aa_3 and cbb_3 type oxidases, meaning either that the targets of Sco differ between species, depending on the oxidases they possess (Frangipani and Haas, 2009; Arunothayanan et al., 2010; Lohmeyer et al., 2012; Thompson et al., 2012; Serventi et al., 2012), or that Sco is hierarchically positioned in such a way that it serves the disparate Cu sites of both oxidase types.

There is evidence for a direct interaction between yeast Sco1 and subunit II of aa_3-COX, and overexpression of Sco1 rescues the aa_3-COX activity of yeast mutants lacking Cox17. These results are the sole basis for a proposed model by which Sco proteins aid the assembly of aa_3-type oxidases by transferring copper directly to the Cu_A center (Robinson and Winge, 2010). Contradictorily, direct transfer of copper from Sco1 to the Cu_A center of the ba_3-type oxidase of *Thermus thermophilus* was not observed, leading to the proposal of an alternative model by which Sco proteins reduce a disulfide bridge within the Cu_A-binding site of subunit II, allowing insertion of Cu by a newly discovered, periplasmic chaperone PCu_AC (Abriata et al., 2008).

In some bacteria (see earlier), Sco proteins are important for the assembly of cbb_3-type COX even though these oxidases lack the Cu_A center and possess only the Cu_B center. In *R. capsulatus*, a species that lacks aa_3-type COX and instead uses a cbb_3-type COX and a quinol oxidase as terminal oxidases (Thöny-Meyer et al., 1994; Ekici et al., 2012a), deletion of the Sco homolog SenC results in reduced cbb_3 COX activity that is restorable by the addition of copper (Lohmeyer et al., 2012). Copper-dependent rescue of cbb_3 activity was also observed in a *senC* mutant of *Pseudomonas aeruginosa* (Frangipani and Haas, 2009). The ability of copper to rescue cbb_3 activity of *sco* mutants supports a possible role for Sco in the delivery of copper to the Cu_B site of cbb_3 COX. *In vitro* cross-linking has shown that SenC of *R. capsulatus* may directly interact with the cbb_3 oxidase during biosynthesis. Delivery of copper to the Cu_B center is likely to involve a different mechanism compared to that for delivery to the Cu_A center, because the Cu_B center is deeply buried within the hydrophobic membrane unlike Cu_A that is located on a surface-exposed, periplasmic domain of subunit II. Further chaperoning proteins may therefore be involved in the delivery to the disparate copper sites (see Sections 29.5.5–29.5.7).

In *B. japonicum*, the Sco homolog ScoI binds copper *in vitro*, and the thiols of two cysteine residues, numbers 74 and 78, were shown to be essential for binding (Bühler et al., 2010). Deletion of the *scoI* gene blr1131 results in loss of aa_3-type cytochrome oxidase assembly and activity, and also appears to affect assembly/activity of the cbb_3 oxidase, but only when *B. japonicum* was examined in the symbiotic state (Serventi et al., 2012). A similar condition-dependent phenotype was observed for *pcuC* mutants, as will be discussed next.

29.5.5 PcuC

B. japonicum PcuC is a homolog of PCu_AC, a copper chaperone first identified by Banci and coworkers in a search of the gene neighborhood of *scoI* for new proteins involved in copper delivery to cytochrome *c* oxidase (Banci et al., 2005). The PCu_AC family of proteins are periplasmic and share a common metal-binding motif $H(M)X_{10}MX_{21\text{-}22}HXM$. This motif is located within a soluble C-terminal cupredoxin-like fold and binds to a single Cu(I) via the methionine and histidine side chains (Banci et al., 2005). The solution structures have been determined for both *Deinococcus radiodurans* (Banci et al., 2005) and *Thermus thermophilus* PCu_AC in both the apo-bound and Cu(I)-bound forms (Abriata et al., 2008). PCu_AC does not have a homolog in mitochondria, but it has been suggested that it performs a similar role to the mitochondrial protein Cox17. Cox17 is a soluble intermembrane protein proposed to transfer copper to Sco1 and Cox11 for assembly of the Cu_A and Cu_B centers, respectively, of cytochrome *c* oxidase (Horng et al., 2004; Banci et al., 2007, 2008).

In *B. japonicum*, there are two genes for homologs of PCu_AC: *pcuC* and blr7088. The gene *pcuC* is contained within an operon of five genes (*pcuABCDE*) (bll4882-4878) that are upregulated under copper starvation (Serventi et al., 2012). Genes *pcuA* and *pcuD* are predicted to encode soluble periplasmic proteins, and *pcuB* and *pcuE* are predicted to encode membrane integral proteins (Serventi et al., 2012). Gene *pcuA* contains a CXXC sequence and is conserved among the rhizobia, *pcuD* is of unknown function, *pcuB* encodes a TonB-dependent receptor, and finally *pcuE* encodes a protein similar to *B. subtilis* YcnJ or a fusion of CopC/CopD (Serventi et al., 2012). Blr7088 is located adjacent to the *nirK* gene (blr7089) encoding nitrite reductase. Despite being located next to *nirK*, mutation of blr7088 has neither any effect on anoxic growth with nitrate as the terminal electron acceptor nor any effect on aa_3-type cytochrome *c* oxidase activity or symbiotic nitrogen fixation (Serventi et al., 2012).

Deletion of the *pcuABCDE* operon results in a pleiotrophic phenotype compared to wild type, with impaired growth under copper starvation, impaired growth under conditions of anoxic nitrate respiration, a 75% decrease in symbiotic nitrogen-fixation activity, and a complete lack of activity of the aa_3-type cytochrome *c* oxidase (Serventi et al., 2012). Surprisingly, *pcuC* is plainly responsible for all of the aforementioned phenotypes (except perhaps the defect in growth under anoxic nitrate respiration where the exact contribution of each gene to this phenotype could not be clearly determined) (Serventi et al., 2012). The lack of a clear copper phenotype for the other genes does not exclude them from copper metabolism as they may be redundant in function or only important in environmental niches where copper concentrations are too low to be effectively mimicked by laboratory media.

Similar to other members of the PCu_AC family, PcuC binds a single Cu(I) in a 1:1 ratio (Serventi et al., 2012). The impaired nitrate respiration of the *pcuC* mutant suggests that PcuC may also be involved in the delivery of copper

to nitrite reductase NirK (Serventi et al., 2012). The importance of PcuC for symbiotic nitrogen fixation also points to PcuC being involved in copper delivery to the Cu_B center of the symbiotic cbb_3-type cytochrome oxidase. Enigmatically, membranes prepared from a *pcuC* mutant grown anaerobically with nitrate as the terminal electron acceptor (a situation under which nearly all cytochrome *c* oxidase activity is due to the cbb_3 oxidase) show wild-type levels of oxidase activity while those prepared from root-nodule bacteroids show strongly impaired activity (Serventi et al., 2012). This implies that PcuC plays an important role in copper delivery to the cbb_3 oxidase but only during endosymbiosis. How is this possible? It may be that PcuC is important for cbb_3 activity under both growth conditions, but that the copper available in the denitrification growth medium (0.2 μM; below this level even wild-type cells do not grow) was high enough to bypass the need for PcuC (Serventi et al., 2012). Increasing the copper concentration within the plant growth medium does not successfully restore cbb_3 oxidase activity of *pcuC* mutant bacteroids, but it is difficult (if not technically insurmountable) to determine how much copper is available to *B. japonicum* symbionts growing within nodules, how it is supplied by the plant, and whether increasing copper in the plant growth medium would increase copper availability for the bacteroid (Serventi et al., 2012).

The involvement of PCu_AC in cbb_3 oxidase assembly is not unique to *B. japonicum*. In *R. sphaeroides*, Thompson et al. have recently shown that PCu_AC is involved in the formation of both the Cu_A center of the aa_3-type oxidase and the Cu_B center of the cbb_3 oxidase (Thompson et al., 2012). They also found that, to a lesser degree, and despite the presence of Cox11, PCu_AC is also involved in formation of the Cu_B center of the aa_3-type oxidase. They have proposed that Cu_B is predominantly assembled by Cox11, but that PCu_AC helps to supply copper under low-copper conditions (Thompson et al., 2012). The exact copper center (Cu_A or Cu_B of the aa_3-type COX) that *B. japonicum* PcuC is supplying copper is currently unknown, and it is uncertain whether PcuC plays a direct role in construction of the copper centers or, alternatively, whether it supplies copper to another downstream protein that carries out this role. If PcuC does supply copper to another downstream chaperone, then ScoI would be a likely candidate. Future work must determine whether or not this is indeed the case.

29.5.6 CoxG/Cox11

CoxG, also termed CtaG in some Alphaproteobacteria, is a homolog of the mitochondrial protein Cox11 (Cox11p in yeast) and is involved in copper trafficking to the Cu_B site of aa_3 COX subunit I. For simplicity, we refer to all eukaryotic homologs as Cox11 and all prokaryotic homologs as CoxG in this review. (Note that CoxG/CtaG/Cox11 is unrelated to CtaG of *Bacillus subtilis*, which is involved in formation of a caa_3-type cytochrome (Bengtsson et al., 2004)).

Cox11 was first revealed to be required for the assembly of cytochrome *c* oxidase in yeast, *Saccharomyces cerevisiae*, where mutants lacking Cox11 exhibit a deficiency in cytochrome *c* oxidase activity (Tzagoloff et al., 1990). Subsequently in *R. sphaeroides*, it was shown that cytochrome aa_3 isolated from a Δ*coxG* mutant specifically lacked the Cu_B center (Hiser et al., 2000). The cytochrome *c* oxidase still contained both hemes but electron paramagnetic resonance spectroscopy showed that heme a_3, associated with the Cu_B binuclear center, was misaligned (Hiser et al., 2000).

The exact timing of Cu_B site formation is still unclear, but because Cu_B is deeply buried within subunit I in the cytoplasmic membrane/inner mitochondrial membrane and in a binuclear complex with heme A_3, it is presumed that the formation of the Cu_B center takes place at around the same time as insertion of heme A_3. In *S. cerevisiae*, Cox11 was found to be associated with the mitochondrial translation machinery, suggesting that Cu_B may be formed by a transient interaction between Cox11 and the nascent Cox1 protein during insertion of Cox1 into the inner mitochondrial membrane (Khalimonchuk et al., 2005). Additional evidence for an association of Cox11 with the translation machinery comes from *Schizosaccharomyces pombe* where Cox11 is encoded as a fusion (but later separated by proteolytic cleavage) with ribosomal component Rsm22 (Carr et al., 2005).

Cox11/CoxG is anchored to the inner mitochondrial membrane/bacterial cytoplasmic membrane by a single N-terminal transmembrane domain and has a soluble C-terminal domain that faces the intermembrane space/periplasm (Tzagoloff et al., 1990; Banci et al., 2004; Carr et al., 2005). The C-terminal domains of all Cox11/CoxG proteins are highly conserved.

Biochemical analysis of yeast Cox11 has determined that it is a dimer in both the apo-bound and Cu(I)-bound forms, and that Cox11 binds to one Cu(I) ion per monomer via three thiolate ligands (Carr et al., 2002). The two copper ligands in the Cox11 dimer form a binuclear cluster, and mutation of any of the three conserved Cys residues, two within a highly conserved CFCF motif in the middle of the C-terminal domain of Cox11 and a third at the C-terminus of the transmembrane domain, was found to decrease Cu(I) binding and oxidase activity (Carr et al., 2002; Banting and Glerum, 2006).

The solution structure of the monomeric, metal-free soluble domain of *Sinorhizobium meliloti* CoxG (apo-CoxG) was determined, and Cu(I) binding to CoxG was confirmed to involve formation of a binuclear Cu(I) cluster but composed of only two cysteines in the CFCF motif (Banci et al., 2004). The third conserved cysteine was proposed to form an intermolecular disulfide bond between CoxG monomers essential for function (Banci et al., 2004). Recently, contrary

to earlier reports, Thompson et al. found that in *Rhodobacter sphaeroides*, this cysteine is not required for dimerization of CoxG or for binding of copper. They instead propose that this cysteine is involved in transfer of Cu(I) from the copper binding site of CoxG to the Cu_B site of subunit I (Thompson et al., 2010).

In *B. japonicum*, *coxG* is located within the *cox* operon encoding cytochrome *c* oxidase subunits I–III (*coxA–C*), heme O synthase (*coxE*), and Surf1c (*shb1*) (Fig. 29.1). A knockout mutation of *coxG* results, as one would expect, in loss of cytochrome aa_3 assembly and activity, but surprisingly does not affect symbiotic nitrogen fixation that depends on the activity of cytochrome cbb_3 (Bühler et al., 2010). If *B. japonicum coxG* is required for cytochrome aa_3 assembly, then why is it not also an important chaperone for assembly of the Cu_B-containing cytochrome cbb_3?

29.5.7 FixGHIS

The *fixGHIS* gene cluster (termed *ccoGHIS* in *R. capsulatus* and *rdxBHIS* in *R. sphaeroides*) is required for assembly of the cbb_3 oxidase in rhizobia and *R. capsulatus/sphaeroides*. In most bacteria in which the *fixGHIS* gene cluster has so far been identified, it is located immediately downstream of the *fixNOQP* operon for the cbb_3 oxidase (Kahn et al., 1989; Mandon et al., 1993; Preisig et al., 1993; Thöny-Meyer et al., 1994; Ducluzeau et al., 2008). The *fixGHIS* gene cluster was first characterized in *Sinorhizobium meliloti* where transposon disruption of the *fixG*, *fixH*, *or fixI* genes gave the same defective symbiotic nitrogen fixation (Fix^-) phenotype as mutants of the *fixNOQP* genes (Kahn et al., 1989). In *B. japonicum*, the mutation of *fixGHIS* results in the Fix^- phenotype and in decreased cytochrome oxidase activity measured in cells grown under micro-oxic conditions (Preisig et al., 1996b). Membranes isolated from the *B. japonicum fixGHIS* mutant have only trace amounts of the cbb_3 oxidase showing that one or more of the FixGHIS proteins are important for its assembly (Preisig et al., 1996b). In *Azorhirobium caulinodans*, *fixGHI* is not essential for symbiotic nitrogen fixation (Mandon et al., 1993), but it was later discovered that this species utilizes two separate oxidases for symbiotic nitrogen fixation: a cbb_3 oxidase and a *bd* oxidase (loss of only one oxidase reduces nitrogen fixation by 20–30%) (Kaminski et al., 1996). In *R. sphaeroides*, genetic disruption of the FixG homolog RdxA established that it was not essential for nitrogen fixation (Neidle and Kaplan, 1992). However, unlike *B. japonicum* and *S. meliloti*, *R. sphaeroides* possesses a second homolog of *fixG* that may functionally compensate for loss of RdxA (Neidle and Kaplan, 1992).

The *fixGHIS* gene cluster encodes four predicted integral membrane proteins (Kahn et al., 1989). FixG is predicted to have five transmembrane helices, and contains two cysteine-rich motifs like those found in bacterial ferredoxins for coordination of iron-sulfur [4Fe–4S] clusters. In *R. sphaeroides*, the FixG homolog RdxA is inserted into the inner membrane in such a way that the cysteine-rich motifs project into the cytoplasm (Neidle and Kaplan, 1992). FixG may be involved in a redox process coupled to cation transport catalyzed by FixI (Kahn et al., 1989). Likewise, Preisig et al. proposed that FixG performs an oxidoreductase role based on the resemblance of its cysteine-rich motifs to the [4Fe–4S] cluster-binding motifs of ferredoxin proteins, and that it might be involved in intracellular oxidation of Cu(I) to Cu(II) following transport of Cu(I) across the membrane by FixI (Preisig et al., 1996b).

FixH, aside from two putative transmembrane helices, does not contain any defined motifs, cofactor/metal-binding sites, or homology with any proteins of known function (Kahn et al., 1989; Preisig et al., 1996b). In *R. capsulatus* deletion of the *fixH* homolog *ccoH* caused a large decrease in the amount of cbb_3 oxidase in the membrane despite normal wild-type levels of transcription of the *ccoNOQP* operon (Koch et al., 2000). This suggests that CcoH is important for posttranslational assembly of the cbb_3 oxidase. The function of FixH is still unknown, but Pawlik et al. found that CcoH interacts with CcoP (subunit III) of the cbb_3 oxidase and that it appears to remain tightly associated with CcoP even once the cbb_3 oxidase is fully assembled (Pawlik et al., 2010).

FixI is thought to be important for copper homeostasis due to its homology to the copper uptake protein CopA of *Enterococcus hirae*. CopA is a member of the P-type ATPase family that is involved in transport of heavy metal ions in prokaryotes and eukaryotes (reviewed by Solioz and Stoyanov (2003)). FixI possesses all of the characteristic sequence motifs of the P-type ATPase family including the CXXC motif thought to be the metal-binding site, the TGE motif that forms part of the phosphatase domain, the CPC motif involved in ion transduction, the DKTGT motif containing an aspartyl residue that is the site of phosphorylation, and the VGDG motif that forms part of the kinase domain (Kahn et al., 1989; Preisig et al., 1996b). In *R. capsulatus ccoI* was also found to be required for assembly of the cbb_3 oxidase, and this phenotype could be mimicked by copper starvation of wild-type *R. capsulatus*, suggesting that CcoI may be important for copper transport (Koch et al., 2000). A likely target for FixI/CcoI-mediated copper delivery is subunit I (FixN/CcoN) of the cbb_3 oxidase that contains a Cu_B center. There is evidence that if CcoI is a copper transporter, then it may not be dedicated solely to copper delivery to the cbb_3 oxidase. In *Rubrivivax gelatinosus*, it has recently been reported that the CcoI homolog in this species, CtpA, may be involved in delivery of copper to other Cu-enzymes including the aa_3-type oxidase and nitrous oxide reductase NosZ (Hassani et al., 2010). There is also evidence that more than one transporter is involved

in copper delivery to the cbb_3 oxidase. Recently, a novel major-facilitator superfamily (MFS)-type transporter CcoA was implicated in copper delivery to the cbb_3 COX in *R. capsulatus* (see Section 29.5.8).

FixS contains a putative N-terminal membrane anchor, but does not contain any other obvious sequence motifs and is not similar to any proteins of known function (Kahn et al., 1989; Preisig et al., 1996b). Mutation of *ccoS* in *R. capsulatus* did not affect presence of cbb_3 oxidase in the membrane but rendered it inactive due to absence of the cofactors in CcoN (Kulajta et al., 2006). In summary, there is evidence that at least *fixH*, *fixI,* and *fixS* play crucial roles in the assembly of the cbb_3 oxidase.

29.5.8 CcoA

Recent research in the purple nonsulfur bacterium *Rhodobacter capsulatus* has identified an MFS-type transporter termed CcoA required for assembly of cbb_3-COX (Ekici et al., 2012b). CcoA was identified by screening a library of cbb_3-COX defective mutants for restoration of cbb_3-COX activity by Cu^{2+} supplementation. In the absence of CcoA, cellular copper content was found to be decreased and assembly and activity of cbb_3-COX was defective (Ekici et al., 2012b). Although several homologs of *ccoA* are found in *B. japonicum*, none of them are upregulated under copper starvation. Instead we believe that another gene, bll0889, may encode an ortholog of *ccoA*. Gene bll0889 is predicted to encode an MFS-type transporter and is located within a cluster of seven genes, all upregulated (but none as strongly as bll0889) under extreme copper starvation (Serventi et al., 2012).

29.6 FUTURE PERSPECTIVES

Several burning questions remain to be answered, of which a selection of two is given here:

1. What is the function of the oxygen reductases other than cytochromes aa_3 and cbb_3 in the physiology of *B. japonicum*? Will it be possible to identify specific growth conditions and specific substrates for heterotrophic or lithotrophic oxidation, which require a particular cytochrome/ubiquinol oxidase in a conceptually similar manner as the cbb_3-type oxidase is required for micro-oxic respiration in symbiosis?
2. How is copper taken up into free-living cells or endosymbiotic bacteroids, and how is it delivered to target cuproproteins such as the heme-copper oxidases? Although a number of metallochaperones have meanwhile been identified, the situation is far from being clear with regard to how they cooperate. For example, the intriguing similarity of phenotypes displayed by *tlpA*, *scoI*, and *pcuC* mutants would argue for a concerted action of all three proteins. Are they hierarchically arranged so that copper can be relayed from one protein to the next? Which of the proteins decisively discriminates between the final metallation of the Cu_A and Cu_B centers? More biochemical work, such as establishing a copper transfer assay with purified components *in vitro*, will be needed.

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Chapter 30

Rhizobial Extracytoplasmic Function (ECF) σ Factors and Their Role in Oxidative Stress Response of *Bradyrhizobium japonicum*

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30.1 GENE REGULATION MEDIATED BY σ FACTORS

Bacterial σ factors determine promoter specificity of RNA polymerase (RNAP). They are divided into two structurally and functionally distinct groups, the σ^{54} and the σ^{70} (Wösten, 1998; Gruber and Gross, 2003). Transcription by RNAP containing σ^{54} is initiated from promoters that show high conservation around positions −24 and −14 relative to the transcription initiation site and obligatorily requires an enhancer-binding protein (EBP) and adenosine triphosphate (ATP) hydrolysis (for reviews, see (Ghosh et al., 2010; Bush and Dixon, 2012)). Many genes involved in nitrogen fixation in rhizobia and other diazotrophs are preceded by −24/−14-type promoters and thus transcribed by RNAP associated with σ^{54}.

The σ factors of the σ^{70} type are ubiquitous bacterial proteins comprising two to four structurally and functionally conserved domains (Gruber and Gross, 2003; Paget and Helmann, 2003). Extensive analysis of σ^{70}-type factors has revealed the roles of different domains in promoter recognition and initiation of transcription (Fig. 30.1). For example, domains σ_4 and σ_2 of σ^{70}-type σ factors make contacts with conserved promoter elements around positions −35 and −10, respectively, relative to the transcription initiation site (for reviews, see (Paget and Helmann, 2003; Österberg et al., 2011; Decker and Hinton, 2013)). Four distinct subgroups of σ^{70} factors are defined based on structural and functional features. Sigma factors of groups 1 and 2 possess all four conserved domains and comprise the largest σ factor proteins (ca. 70 kDa). While group 1 consists of essential primary σ factors, groups 2–4 include alternative σ factors required for specific bacterial functions that may or may not be essential. Sigma factors RpoD and RpoS of *Escherichia coli* are classical examples of group 1 and group 2 σ factors, respectively.

The σ factors of group 3 are significantly smaller in size (ca. 20–35 kDa) than those belonging to groups 1 and 2 because they lack domain σ_1 (Fig. 30.1). Members of this group control diverse cellular functions such as sporulation, heat shock protection, and flagella biosynthesis. Prominent examples of group 3 are the *Bacillus subtilis* factor SigH (σ^H) involved in the initiation of sporulation, the *E. coli* heat shock σ factor RpoH, and the *E. coli* FliA σ factor, which directs transcription of flagella and chemotaxis genes.

The largest group among σ^{70}-type factors is group 4, which comprises small σ factors (ca. 20–25 kDa) consisting only of domains σ_4 and σ_2 (Fig. 30.1). Group 4 σ factorsare highly diverse with regard to function and amino acid

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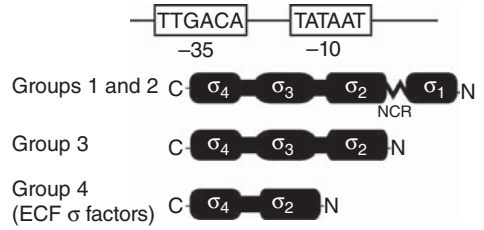

Figure 30.1 Classification and simplified domain architecture of σ^{70}-type σ factors. The role of domains σ_2 and σ_4 in recognition of −10 and −35 promoter elements is indicated. NCR indicates a nonconserved linker region between domains σ_2 and σ_1 of group 1 and 2 σ^{70} proteins. See text for further details.

sequence. Many σ factors of this group respond to signals from the extracytoplasmic compartment, and hence members of group 4 are referred to as extracytoplasmic function (ECF) σ factors. ECF σ factors control genes involved in different cellular functions, such as stress responses, metal homeostasis, virulence-related traits, and cell envelope structure. The number of ECF σ factors varies widely among bacterial species. For example, bacteria belonging to the *Chlamydiae* phylum or *Borrelia* genus lack this type of σ factor, *Staphylococcus* spp. harbor 1, *E. coli* 2, *B. subtilis* 7, *Mycobacterium tuberculosis* 10, *Caulobacter crescentus* 13, *Pseudomonas aeruginosa* 18, and *Streptomyces coelicolor* 50 ECF σ factors (Helmann, 2002; Staroń et al., 2009). ECF σ factors, particularly those present in rhizobia, are the focus of this chapter.

30.2 DIVERSITY AND COMMON FEATURES OF ECF σ FACTORS

Apart from their common structural organization, most ECF σ factors share four features: (i) ECF σ factors in complex with RNAP often transcribe their own gene and thus create a positive-feedback loop. (ii) In general, ECF σ factors control relatively small regulons. (iii) Activity of ECF σ factors is often controlled negatively via protein–protein interaction with an anti-σ factor. Usually, in the absence of stimuli, association of an ECF σ factor with RNAP is prevented by binding to its cognate anti-σ factor. In the presence of an appropriate stimulus, the ECF σ factor is released from the σ–anti-σ factor complex and binds to RNAP. (iv) Genes coding for ECF σ and anti-σ factors often form an operon and thus are tightly coregulated.

The diversity of bacterial ECF σ factors has been addressed only relatively recently in systematic analyses. In a bioinformatics analysis of ECF σ factors retrieved from sequenced bacterial genomes, more than 40 distinct classes of ECF σ factors were defined based on amino acid sequence similarity and domain structure of the associated anti-σ factors (Staroń et al., 2009). The study revealed that ECF σ factors exhibit a conserved domain structure and are ubiquitous in bacterial species while anti-σ factors show a surprising structural diversity. Many (but not all) anti-σ factors consist of a cytoplasmic portion that mediates ECF σ factor inhibition and an inner membrane or periplasmic domain that can sense extracytoplasmic signals. Great diversity of anti-σ factors likely reflects their ability to sense various stimuli and transduce the signals to the cognate ECF σ factors.

30.3 σ FACTORS IN RHIZOBIA

Transcription of many genes involved in nitrogen fixation is under control of a redox-responsive EBP-family protein NifA that acts in concert with RNAP containing σ^{54}. Accordingly, much attention was given to rhizobial σ^{54} factors (also named RpoN) in the pre-genomic era (Ronson et al., 1987; Kullik et al., 1991; Stigter et al., 1993; Fischer, 1994; Michiels et al., 1998a,b; Clark et al., 2001). Notably, several rhizobial species, including *B. japonicum*, *Mesorhizobium loti,* and *Rhizobium etli*, possess two variants of σ^{54} (Table 30.1).

Apart from the σ^{54} factors, rhizobia possess different numbers of σ^{70} factors (Table 30.1). Similar to most bacteria, rhizobial genomes encode one primary σ factor. The primary σ factors of *B. japonicum*, *R. etli*, and *S. meliloti* are very similar to that of *E. coli*, except for an extended σ_1 region that makes rhizobial proteins larger than the *E. coli* ortholog (about 80 kDa instead of 70 kDa) (Rushing and Long, 1995; Luka et al., 1996; Beck et al., 1997).

Unlike *E. coli*, rhizobia do not encode group 2 σ^{70} factors, but they often possess multiple paralogs of group 3 σ factors (Table 30.1). These σ factors, named RpoH according to the *E. coli* heat shock σ factor, display distinct functions and modes of regulation. Of the three *rpoH* genes in *B. japonicum*, $rpoH_2$ is essential for growth but the remaining two *rpoH* genes are dispensable for free-living growth and symbiosis (Narberhaus et al., 1997). In *S. meliloti,* $RpoH_1$ is required for efficient symbiosis while deletion of the other *rpoH* paralog does not lead to any discernible phenotype (Mitsui et al., 2004; Barnett et al., 2012).

While ECF σ factors belonging to group 4 are most abundant among (putative) rhizobial σ factors, comparatively little information is available about their function(s) and mode of regulation (Table 30.2).

In *B. japonicum*, two ECF σ factors EcfS (Bll4928) and EcfG (Blr7797) were shown to be required for an efficient symbiotic interaction with soybean host plants (Gourion et al., 2009; Stockwell et al., 2012). EcfG represents the general stress response σ factor that is regulated by its anti-σ factor NepR (Bsr7796) and its anti-anti-σ factor PhyR (Bll7795). EcfG-dependent gene expression can be induced by nutrient starvation (Francez-Charlot et al., 2009; Gourion et al., 2009). EcfS is negatively controlled by the putative anti-σ factor TmrS, which is cotranscribed with *ecfS*; yet no

Table 30.1 Numbers of σ^{54}- and σ^{70}-type σ factors in six rhizobial species and in *E. coli*

Organism*	Number of (Predicted) σ Factors in Individual Families and Groups					
	σ^{54} Family	σ^{70} Family				
	Total No.	Total No.	Group 1 (SigA, RpoD)	Group 2	Group 3 (RpoHs)	Group 4 (ECFs)
Bradyrhizobium japonicum USDA 110	2	21	1	—	3	17
Bradyrhizobium sp. BTAi1	1	20	1	—	1	18
Mesorhizobium loti MAFF303099	2	22	1	—	1	20
Rhizobium etli CFN 42	2	20	1	—	2	17
Rhizobium leguminosarum bv. viciae 3841	1	14	1	—	2	11
Sinorhizobium meliloti 1021	1	14	1	—	2	11
Escherichia coli†	1	6	1	1	2	2
	RpoN		RpoD	RpoS	RpoH, FliA	RpoE, FecI

*Listed are rhizobial species whose ECF σ factors were analyzed by (Staroń et al., 2009).

†The designation of individual *E. coli* σ factors is indicated below their respective numbers.

Table 30.2 Numbers and classification of ECF σ factors encoded in rhizobial genomes

Organism*	ECF Group Number†										
	11	15	16	18	20	26	29	33	41	42	Unclassified
Bradyrhizobium japonicum USDA 110‡	—	1	2 EcfF EcfS	1	1	3	1	2 EcfQ Blr3042	1	3	2
Bradyrhizobium sp. BTAi1	1	1	2	2	1	2	1	2	—	2	4
Mesorhizobium loti MAFF303099	1	1	1	1	2	3	1	1	1	3	5
Rhizobium etli CFN 42	1	2	1	—	1	4	1	—	1	4	2
Rhizobium leguminosarum bv. viciae 3841	1	1	—	—	1	4	—	—	1	2	1
Sinorhizobium meliloti 1021	—	2	1	—	—	4	1	—	1	1	1

*Listed are rhizobial species whose ECF σ factors were analyzed by (Staroń et al., 2009).

†Group numbers according to the classification of (Staroń et al., 2009).

‡The designation of *B. japonicum* USDA 110 σ factors belonging to ECF group 16 and 33, which are discussed in detail in this chapter, are specified below the respective numbers.

information about the inducing signal(s) is available. More recently, two additional ECF σ factors of *B. japonicum*, EcfQ (Bll1028), and EcfF (Blr3038), were studied, and it turned out that they contribute to the oxidative stress response in this bacterium (Masloboeva et al., 2012; see below).

A general stress response ECF σ factor, RpoE2 (SMc01506), was also studied in *S. meliloti* (see Chapter 40). Deletion of *rpoE*2 had no effect on symbiotic properties and on the tolerance of free-living cells to various stress conditions (Sauviac et al., 2007; Bastiat et al., 2010). In *S. meliloti*, RpoE2 is regulated by the NepR-type anti-σ factors RsiA1 and RsiA2 (SMc01505, SMc04884) and the PhyR-type anti-anti-σ factors RsiB1 and RsiB2 (SMc01504, SMc00794), which are both present as two paralogs (Bastiat et al., 2010). Recently, two additional ECF σ factors of *S. meliloti*, RpoE1, and RpoE4, were characterized and shown to be activated in stationary phase likely by endogenously produced sulfite (Bastiat et al., 2012). An exhaustive mutagenesis approach is currently applied to all *S. meliloti* ECF σ factor genes in the laboratory of S.R. Long, Stanford University, CA, USA [http://www.isv.cnrs-gif.fr/colloque-AK2012/program.html].

In *R. leguminosarum* bv. viciae, the ECF σ factor RpoI is required for synthesis of the vicibactin siderophore and iron uptake. It is similar to ECF-type σ factors PvdS, PfrI, and PdrA of *Pseudomonas* spp. and, to a weaker extent, also to *E. coli* FecI (Yeoman et al., 1999; Carter et al., 2002). The mechanism of how RpoI is regulated has not been unraveled. In the following sections, the role of oxidative stress as an inducer of ECF σ factor-mediated responses is discussed.

30.4 DIVERSITY AND SOURCES OF REACTIVE OXYGEN SPECIES (ROS)

Key biological processes such as respiration and defense reactions are dependent on molecular oxygen (O_2). Molecular oxygen contains two unpaired, spin-aligned electrons in its outer *p* molecular orbitals. Such orbital occupancy enables O_2 to accept electrons or energy to generate type

I or type II reactive oxygen species (ROS), respectively (for review, see (Cadenas, 1989; Ziegelhoffer and Donohue, 2009)). A one-electron reduction of O_2 results in the formation of a superoxide anion radical (superoxide, $O_2^{\bullet -}$). Further reduction by the transfer of a second electron produces a peroxide anion (O_2^{2-}), which exists as hydrogen peroxide (H_2O_2) in biological systems. In turn, H_2O_2 reacts with iron ions (Fe^{2+}) in the Fenton reaction that leads to the formation of hydroxyl radicals ($OH^{\bullet}$). The type II ROS singlet oxygen ($^1O_2$) is produced as a result of energy transfer to O_2. Different ROS have distinct properties such as chemical reactivity, half-life, and solubility (Halliwell and Gutteridge, 1999). Most reactive among ROS are hydroxyl radicals whereas superoxide is much less deleterious, because it does not penetrate membrane bilayers due to its negative charge. Because of the rapid interconversion of different ROS in biological systems, it is difficult to assign specific effects to individual ROS.

While the respiratory chain of aerobic organisms is a major source of type I ROS, type II ROS are predominantly generated by excited chromophores in photosynthetic microorganisms and plants (Imlay, 2013). A mixture of ROS is generated deliberately in numerous biological processes. Among them is the respiratory burst by stimulated phagocytes that generate ROS via NADH oxidase. Likewise ROS can be detected during the initial steps of plant–pathogen and plant–symbiont interactions where ROS not only play a role in host defense reactions but also act as signaling molecules (for reviews, see (Nanda et al., 2010; Puppo et al., 2013)).

30.5 ROS IN THE *RHIZOBIUM*–LEGUME SYMBIOSIS

Rhizobial lipochito-oligosaccharide signal molecules (Nod factors) and initial contact of rhizobial cells with the epidermis of roots induces host defense reactions including production of ROS (H_2O_2, superoxide) and nitric oxide (NO) (for reviews, see (Pauly et al., 2006; Chang et al., 2009; Nanda et al., 2010; Saeki, 2011; Puppo et al., 2013; see Chapter 64)). Unlike the response to pathogens, however, the response to symbiotic rhizobia is transient and eventually suppressed by proficient symbionts (Santos et al., 2001; El Yahyaoui et al., 2004; Kouchi et al., 2004; Zamioudis and Pieterse, 2012). Notably, H_2O_2 seems to act also as a developmental signal for invading bacteria as indicated by the delayed nodulation phenotype of a *S. meliloti* strain overexpressing a catalase gene (Jamet et al., 2003). Thus, at the early stage of the rhizobia–legume interaction, bacterial antioxidant defense reactions are crucial to overcome the oxidative stress imposed by the host (Santos et al., 2000; Bueno et al., 2001).

The threat imposed on rhizobial symbionts by ROS persists also in mature nodules because of the high abundance of leghemoglobin. This plant-derived hemoprotein functions as an oxygen buffer. It maintains the concentration of free oxygen in the low nanomolar range, which protects oxygen-labile nitrogenase from irreversible inactivation and yet enables bacteroids to generate ATP via microaerobic respiration with the specialized FixNOQP cytochrome oxidase that has a high oxygen affinity. Auto-oxidation of leghemoglobin is probably a major source of superoxide in nodules (Puppo et al., 1991; Gunther et al., 2007). Moreover, transition metals that are abundant in nodules in combination with preformed H_2O_2 might generate hydroxyl radicals, ferryl heme proteins, and protein radicals (Becana and Klucas, 1992; Davies and Puppo, 1992; Moreau et al., 1996).

Finally, elevated concentrations of peroxides and protein carbonyls were reported to occur during natural and stress-induced nodule senescence possibly due to increased concentrations of free iron that serves as a catalyst for ROS generation (Escuredo et al., 1996; Gogorcena et al., 1997; Evans et al., 1999; Becana and Klucas, 1992; Mathieu et al., 1998).

30.6 ROS DETOXIFICATION IN THE *RHIZOBIUM*–LEGUME SYMBIOSIS

ApartRhizobia possess multiple enzymatic antioxidant defense systems with some of them also required for the development and/or proper functioning of symbiosis (for reviews, see (Becana et al., 2010; Puppo et al., 2013)). *S. meliloti* possesses two superoxide dismutase enzymes (SodA and SodC; (Santos et al., 1999; Santos et al., 2000; Flechard et al., 2009)) plus three catalases, that is, two monofunctional hydroperoxidases (KatA, KatC) and a bifunctional catalase-peroxidase (KatB) (Hérouart et al., 1996; Ardissone et al., 2004). In this bacterium, deletion of an individual catalase gene does not cause a symbiotic defect, but double-deletion mutants lacking KatA plus KatC or KatB plus KatC are impaired in nodule formation and nitrogen fixation (Sigaud et al., 1999; Jamet et al., 2003). Reduced nitrogen-fixation activity was also observed when the symbiotic properties of a *katG, prxS* (encoding a peroxiredoxin) double mutant of *Rhizobium etli* were tested on its host plant *Phaseolus vulgaris* (Dombrecht et al., 2005).

Apart from ubiquitous ROS detoxifying systems, rhizobial genomes encode additional enzymes that might play a role in ROS elimination. For example, the *S. meliloti* genome encodes three alkyl hydroperoxide reductases that also might use H_2O_2 as a substrate (Seaver and Imlay, 2001). In addition, *S. meliloti* secretes a putative chloroperoxidase (Smc01944) upon exposure to various hydroperoxides, and expression of a peroxiredoxin-encoding gene (*prxS*) is induced during symbiosis (Barloy-Hubler et al., 2004; Dombrecht et al., 2005).

With regard to mechanisms regulating oxidative stress response in rhizobia, only limited information is available. In *S. meliloti*, transcription factor (TF) OxyR controls expression of two (*katA* and *katB*) of the three catalase genes (Jamet et al., 2005; Luo et al., 2005)) while ECF σ factor RpoE2 is needed for synthesis of superoxide dismutase SodC and

the third catalase KatC (Flechard et al., 2009). Likewise, the *S. meliloti* OhrR TF negatively regulates expression of the symbiotically dispensable organic hydroperoxide resistance gene *ohr*, and possibly also that of the secreted peroxidase expression of above-mentioned Smc01944 ((Barloy-Hubler et al., 2004; Fontenelle et al., 2011)). In *R. etli*, regulation of catalase gene *katG* might be similar as in *E. coli.* Adjacent to *katG*, an OxyR-like regulatory protein is encoded and the *katG* promoter region contains sequence motifs characteristic of OxyR-binding sites (Vargas Mdel et al., 2003). By contrast, synthesis of KatG in *B. japonicum*, which is required for detoxification of H_2O_2 in aerobically growing cells, does not require the OxyR transcriptional activator (Panek and O'Brian, 2004).

30.7 REACTIVE OXYGEN SPECIES (ROS)-INDUCIBLE ECF σ FACTORS OF *Bradyrhizobium japonicum*

Global transcription profiling of *B. japonicum* cells grown microoxically (0.5% O_2 in N_2) and stressed with hydrogen peroxide revealed 225 regulated genes of which more than 10% have predicted regulatory functions (Fig. 30.2; (Masloboeva et al., 2012)).

This suggests a complex structure of the regulatory network that underlies the oxidative stress response in this bacterium. Within the subset of regulatory genes are two ECF σ factor genes, *ecfQ* (bll1028) and *ecfF* (blr3038), both of which are highly induced not only by hydrogen peroxide but also after exposure to singlet oxygen. Further studies revealed that *ecfQ* and *ecfF* mutants show enhanced sensitivity to singlet oxygen but not to other ROS. Neither *ecfQ* nor *ecfF* is required for an effective symbiosis of *B. japonicum* with different host plants, which is in line with bacteria

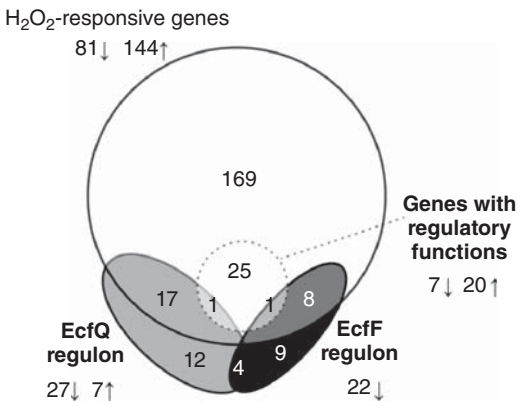

Figure 30.2 Venn diagram representing H_2O_2-responsive genes of *B. japonicum* cells and the genes of (minimally overlapping) EcfQ and EcfF regulons, all determined with cells grown under microoxic conditions. The dashed circle includes H_2O_2-responsive genes with predicted regulatory functions such as various transcription activators or ECF σ factors EcfQ and EcfF. Numbers of downregulated (↓) and upregulated (↑) genes are indicated.

being challenged by superoxide and hydrogen peroxide, but not singlet oxygen, during infection and symbiosis.

Mechanisms controlling expression and activity of EcfQ and EcfF are different. Unlike with *ecfF*, no (putative) anti-σ factor is encoded adjacent to *ecfQ*, and thus it is not known whether activity of EcfQ is controlled at the protein level. Transcription of *ecfQ* might be controlled by a yet to be identified TF, which may bind to conserved DNA motifs located upstream of the transcription start site. By contrast, EcfF is regulated in a manner typical for Ecf σ factors. It drives transcription of its own gene, and its activity is negatively regulated via direct interaction with the transmembrane anti-σ factor OsrA, which is encoded in the *ecfF-osrA* operon. Under nonstress conditions, EcfF activity is inhibited by binding to OsrA. Upon ROS exposure, EcfF is released from the complex thereby becoming available for association with RNAP core enzyme. Cysteine residue 179 of OsrA was shown to be crucial for EcfF activation and thus is an attractive candidate for sensing oxidative stress, yet the molecular details of this process remain to be unraveled (Fig. 30.3).

Using microarray analysis, we determined putative target genes of EcfF and EcfQ in cells grown microoxically. It turned out that both σ factors control rather small, largely distinct sets of genes (Fig. 30.2). The combined EcfQ and EcfF regulons include 13% of the members of the H_2O_2-responsive regulon. Transcriptional control of other H_2O_2-responsive genes outside of the EcfQ-/Ecf-regulons is likely mediated by TFs identified in the H_2O_2-responsive regulon. An example is the *B. japonicum* regulatory protein $FixK_2$, which was shown to be modulated posttranscriptionally by H_2O_2 (Mesa et al., 2009).

Although the regulon of EcfQ is functionally rather undefined (almost 70% of its members are hypothetical or unknown proteins), more than 70% of the proteins encoded by members of the EcfF regulon are functionally annotated. Remarkably, three (Bll5855, Blr7043, and Blr7044) of five methionine-sulfoxide (MetSO) reductases encoded in the *B. japonicum* genome belong to the EcfF regulon. Two of them (Bll5855, Blr7044) are cytoplasmic enzymes, while the third (Blr7043) is predicted to localize to the periplasm (Fig. 30.3). Notably, ECF σ factor-dependent genes for MetSO reductases have been identified previously in two Betaproteobacteria, *Neisseria gonorrhoeae* (Gunesekere et al., 2006) and *Neisseria meningitidis* (Hopman et al., 2010).

The function of MetSO reductases as antioxidant repair enzyme is well documented for a variety of bacterial species (see (Moskovitz, 2005) and references therein). Repair of oxidized methionines by MetSO reductases depends on electron donors which, under physiological conditions, are thioredoxin-like proteins (for review, see (Ezraty et al., 2005)). Accordingly, for repair of MetSOs in the periplasm, additional components are required to transfer electrons across the cytoplasmic membrane and deliver them to MetSO reductases functioning in the periplasm. A well-characterized example of such an electron transfer system is the DsbDC pair of proteins of *E. coli*, which is

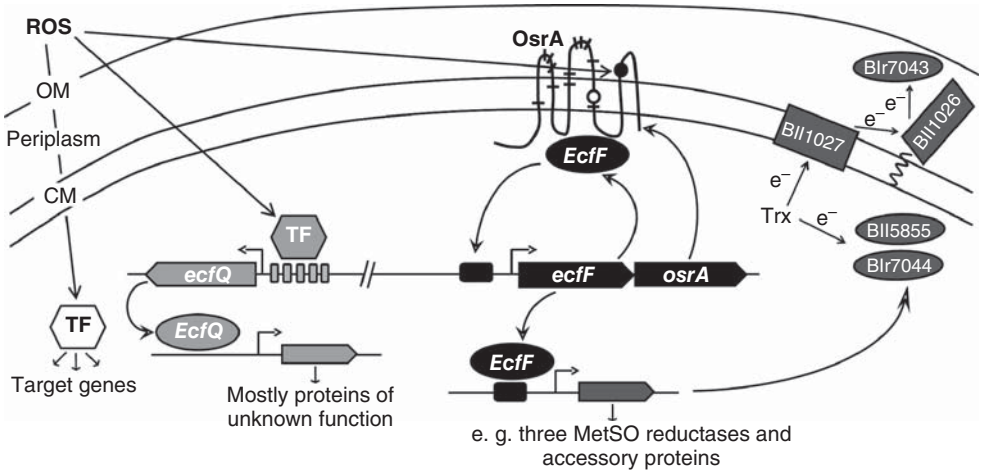

Figure 30.3 The role of ECF σ factors EcfQ and EcfF in the oxidative stress response of *B. japonicum*. The model summarizes the current knowledge about regulation by the two ECF σ factors, both of which contribute to the singlet oxygen tolerance of *B. japonicum*. Both σ factors are induced by reactive oxygen species (ROS). Transcriptional activation of *ecfQ*, which lacks an associated anti-σ factor gene, is postulated to be mediated by a hypothetical transcription factor (TF; gray hexagon) that binds to conserved DNA motifs in the *ecfQ* promoter region (gray boxes). Members of the EcfQ reglon are functionally largely unknown. EcfF is encoded in an autoregulated operon together with the gene for its cognate anti-σ factor OsrA whose predicted transmembrane topology is depicted. OsrA comprises a striking accumulation of methionine residues (bars), which may become oxidized by ROS and thus could be involved in sensing. In fact, a role in H_2O_2 sensing was shown for cysteine 179 (solid circle) while cysteine 129 (open circle) is needed for interaction with EcfF. Upon exposure to ROS, OsrA-bound EcfF is released and associates with RNA polymerase core to initiate transcription from EcfF-dependent promoters (black rectangles). Among EcfF target genes are those of three methionine sulfoxide (MetSO) reductases (Bll5855, Blr7043, Blr7044) and accessory proteins (Bll1026, Bll1027) involved in electron transfer across the cytoplasmic membrane to the periplasmic MetSO reductase. In addition to *ecfQ* and *ecfF*, more than 20 transcription factor genes are induced by ROS, whose products (TF; white hexagon) in turn activate the respective target genes.

needed for reduction or isomerization of disulfide bonds in the periplasm (Kadokura and Beckwith, 2010). Given that bll1026 and bll1027 are members of the EcfF regulon and encode a membrane-anchored periplasmic thioredoxin and an integral membrane protein with a DsbD β core domain, respectively, it is tempting to speculate that these proteins mediate electron transfer from a cytoplasmic electron donor across the membrane to the periplasmic MetSO reductase Blr7043 (Fig. 30.3).

30.8 PARALOGS AND ORTHOLOGS OF ECFF AND ECFQ

According to the classification of ECF σ factors by Staroń and coworkers (Staroń et al., 2009), EcfQ and EcfF of *B. japonicum* belong to groups ECF33 and ECF16, respectively (Table 30.2). Based on the (limited) number of rhizobial species included in the analysis, group 33 ECF σ factors are less prevalent in rhizobia than members of group 16. *B. japonicum* and the related photosynthetic *Bradyrhizobium* sp. BTAi1 each encode two paralogs of EcfQ and EcfF (Blr3042 and EcfS, respectively, in *B. japonicum*). Moreover, in both species, the genes for the group 16 σ factors (EcfF and EcfS) are followed by genes encoding transmembrane anti-σ factors (OsrA and TmrS in *B. japonicum*). By contrast, among the group 33 ECF σ factors, only one paralog comes as a cognate anti-σ–σ-factor pair (Blr3042 and Blr3043 in *B. japonicum*), whereas no anti-σ factor is associated with the second paralog (EcfQ). This may indicate a greater regulatory heterogeneity within group 33 σ factors than in group 16; yet more studies are needed to validate this hypothesis.

The increased singlet sensitivity of *B. japonicum* mutants lacking either EcfQ or EcfF indicates that these σ factors cannot replace each other in this aspect. The same applies to their paralogs Blr3042 and EcfS, which, however, is not surprising because neither of them is induced by oxidative stress. Likewise, EcfF cannot take over the crucial symbiotic role of its paralog EcfS (Stockwell et al., 2012), which is in agreement with the minimal overlap of the respective regulons (Masloboeva et al., 2012). The significance of singlet oxygen tolerance is probably more obvious for the photosynthetic bacterium *Bradyrhizobium* sp. BTAi1 because photosynthesis is a major source of singlet oxygen. Accordingly, it would be interesting to determine the photosynthetic and symbiotic phenotype of *Bradyrhizobium* sp. BTAi1 mutants lacking the orthologs of EcfQ and/or EcfF.

In conclusion, it becomes obvious that currently the genetic and functional diversity of rhizobial ECF σ factors is

only poorly explored. More systematic analyses are needed to get better insight into the complexity of the regulatory networks, which is reminiscent of the diversity in regulatory circuits that control nitrogen fixation and accessory symbiotic genes in rhizobia. Regarding *B. japonicum* EcfQ and EcfS, the elucidation of sensing and signaling mechanisms and the assignment of functions to target genes are obvious, challenging goals of future research.

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Chapter 31

Role of the Bacterial BacA ABC-Transporter in Chronic Infection of Nodule Cells by *Rhizobium* Bacteria

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31.1 INTRODUCTION

Plants can obtain nitrogen through associations with a variety of nitrogen-fixing bacteria. These nitrogen fixers can be loosely associated rhizosphere bacteria, endophytes that are living at low density inside plant tissues, or nodule-inducing bacteria that colonize plants at very high density. Although only a marginal fraction of the total nitrogen needs of plants can be obtained through the first two types of associations, nodulation can support all or a least a major part of its needs. This ability relies on the intimate contact between thousands of intracellular (endosymbiotic) bacteria and their host cells in the root nodules, the symbiotic nodule cells. Mature legume nodules contain several thousand symbiotic cells, each harboring thousands of intracellular, nitrogen-fixing rhizobia called bacteroids. The cytosolic space of the symbiotic cells is *quasi* entirely filled with bacteroids. These symbiotic cells are adapted to the symbiosis, to the metabolic exchange with the nitrogen-fixing rhizobia, and to the accommodation of this large bacterial population. Thus, the symbiotic cells need to maintain a homeostatic interaction with this large bacterial population, whereby elimination of the endosymbionts by defense reactions or, on the contrary, uncontrolled bacterial proliferation and collapse of the host cells have to be avoided. Because the symbiotic nodule cells are at the heart of the

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Rhizobium–legume symbiosis, the mechanisms to reach homeostasis in the symbiotic cells as well as the bacterial adaptations required for their maintenance are now the subject of intense research.

The differentiation of the symbiotic nodule cells from their dividing progenitor cells in the nodule meristem involves genome doublings by endoreduplication cycles resulting in polyploid, giant symbiotic cells. The rhizobia proliferate in the infection threads, which are first initiated in root hairs and then ramify through the growing nodule. Differentiating host cells are penetrated by infection threads and bacteria are released from them through an endocytosis-like process, leaving the intracellular bacteria in membrane-enclosed organelle-like structures called symbiosomes. The symbiosome bacteria differentiate subsequently to nitrogen-fixing bacteroids. These bacteroids have a specific physiology and metabolism adapted to the symbiotic life and nitrogen fixation, which are dramatically different from those of a free-living bacterium. This differentiated state is obtained by a global transcriptome switch, which is primarily regulated by the rhizobial two-component regulator FixLJ. FixLJ is a sensor of the low-oxygen concentration that is prevalent in nodules (Kereszt et al., 2011).

In addition, the differentiation of bacteroids is often, but not always, accompanied by a morphological and cytological metamorphosis whereby the bacteroid cell becomes enlarged, its envelope fragilized, and its genome amplified and condensed. Three different bacteroid morphotypes have been described (Sen et al., 1986; Vasse et al., 1990; Mergaert et al., 2006; Bonaldi et al., 2011): elongated bacteroids which are sometimes also branched (designated below as the E-morphotype), enlarged spherical bacteroids (S-morphotype), and finally bacteroids that remain unmodified and have a rod-shaped morphology similar to free-growing rhizobia (U-morphotype). The metamorphosis to E- or S-morphotype bacteroids takes place in the symbiotic cells after the endocytotic release of rod-shaped *Rhizobium* bacteria in symbiosome vesicles (Vasse et al., 1990; Bonaldi et al., 2011). *Rhizobium* strains that can nodulate legumes of different clades adopt a bacteroid morphotype that is determined by the host. This indicates that the bacteroid morphotype is controlled by the host plant and results from host factors that induce the bacteroid metamorphosis (Sen et al., 1986; Mergaert et al., 2006; Bonaldi et al., 2011; see Chapter 28).

In *Medicago* and related legumes of the inverted repeat-lacking clade (IRLC), which form E-type bacteroids, a class of peptides named NCR (nodule-specific cysteine-rich) peptides control the bacteroid differentiation (Van de Velde et al., 2010). The NCR gene family has been identified in all investigated legume species of the IRLC clade but not in other plant species outside of this clade. Genome and transcriptome analyses have demonstrated that these genes are lacking in the non-IRLC legumes such as *Lotus japonicus*, *Glycine max*, or *Phaseolus vulgaris* (Mergaert et al., 2003; Alunni et al., 2007). A peculiar feature of the family in *Medicago truncatula* is that it is composed of about 600 genes that are all exclusively expressed in nodules (Mergaert et al., 2003; Alunni et al., 2007; Young et al., 2011). Within nodules, the NCR peptides are only produced by the infected symbiotic cells and are transported to the bacteroid-containing symbiosomes (Fig. 31.1). In the *M. truncatula dnf1* mutant that cannot transport NCR peptides anymore to the symbiosomes, bacteroid differentiation is aborted (Fig. 31.1) (Van de Velde et al., 2010; Wang et al., 2010). NCR peptides can induce typical features of E-morphotype bacteroids *in vitro* on cultured *Rhizobium* or *in planta* when expressed in transgenic *L. japonicus* plants, which form usually U-morphotype bacteroids (Van de Velde et al., 2010; Haag et al., 2011). Some NCR peptides accumulate to a significant extent in the cytosol of mature bacteroids (Van de Velde et al., 2010), suggesting that these peptides may have additional functions other than inducing the morphological transformation, and notably, it has been suggested that these intracellular NCR peptides may affect the bacteroid metabolism (Kereszt et al., 2011).

However, NCR peptides are similar to the defensin type of antimicrobial peptides (AMPs), and some NCR peptides have been found to have antimicrobial activity, killing *Rhizobium* when applied at high concentration. To counteract the antimicrobial activity of the NCR peptides, *Sinorhizobium meliloti*, the symbiont of *Medicago*, requires the BacA protein. In the absence of this protein, the bacteroids do not differentiate and are immediately killed by the NCR peptides in the nodule as soon as they are released in the symbiosomes (Haag et al., 2011). Here, we report on the current state of the art on the role of BacA and its homologs in other bacteria.

31.2 *BacA* IS REQUIRED IN LEGUMES FORMING E- OR S-MORPHOTYPE BACTEROIDS BUT NOT IN OTHER LEGUMES

The first rhizobial *bacA* gene was identified in a screen of *S. meliloti* mutants inducing ineffective (Fix^-) nodules on *Medicago sativa* (Glazebrook et al., 1993). In *Medicago* nodules formed by the *S. meliloti bacA* mutant, the bacteria infect the plant cells but do not elongate into the typical E-morphotype shape, and the rod-shaped bacteria in the symbiosomes quickly degenerate and lyse (Figs. 31.1 and 31.2) (Glazebrook et al., 1993; Maunoury et al., 2010; Haag et al., 2011). The gene's name reflects its role in bacteroid differentiation.

More recently, *bacA* genes in other *Rhizobium* species have also been investigated. It has been demonstrated

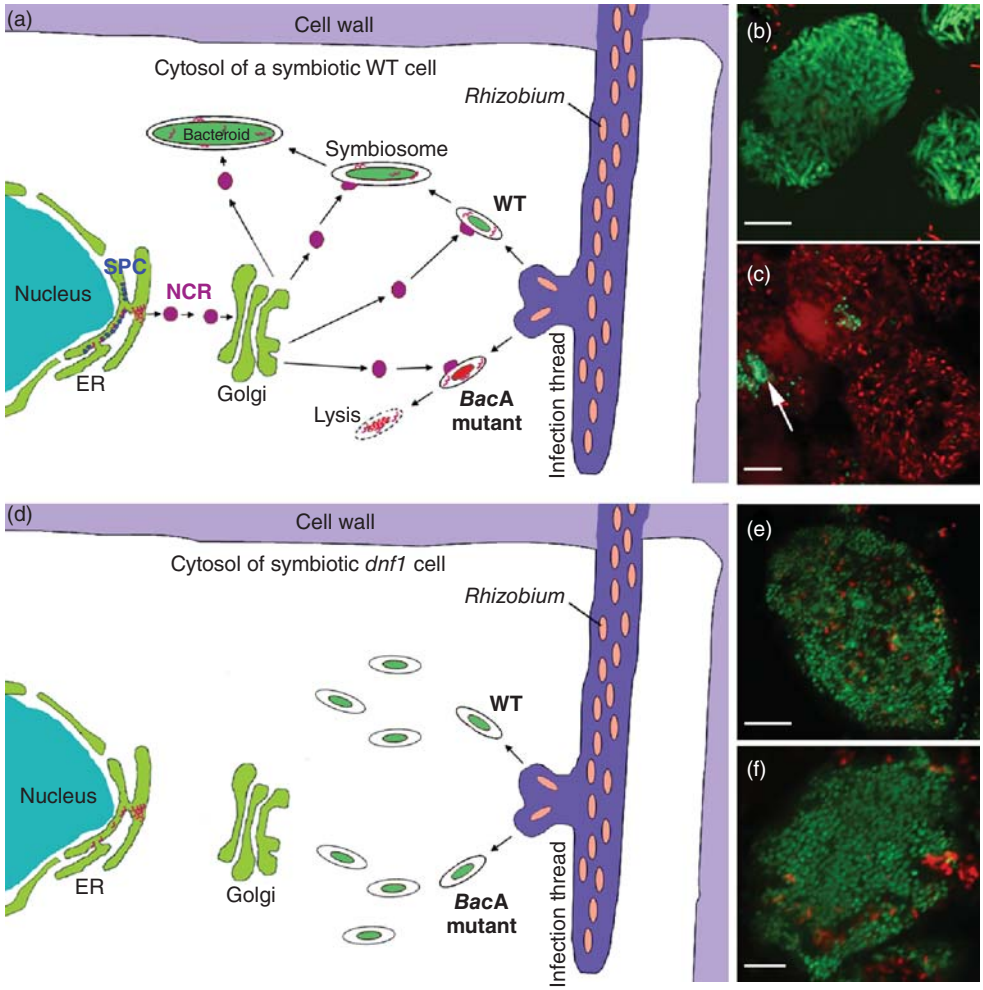

Figure 31.1 BacA is required for survival in the symbiotic nodule cells. (a) In a *M. truncatula* wild-type nodule cell, the *S. meliloti* wild-type (WT) and *bacA* mutant bacteria are released from the infection threads into membrane-bound symbiosomes. The bacteria are then challenged with host NCR peptides (purple), which are synthesized on the endoplasmic reticulum (ER) and transported to the symbiosomes via vesicle transport and a symbiotic nodule-cell-specific secretory pathway. This pathway requires an ER-located nodule-specific SPC containing the DNF1 subunit. The NCR peptides mediate the differentiation of the WT strain into a nitrogen-fixing bacteroid (green bacteroids), resulting in the chronic infection. In contrast, the *bacA*-deficient mutant is hypersensitive toward the host NCR peptides and is killed and then lysed (red symbiosome bacteria) rather than forming the chronic infection. (b) WT *S. meliloti* in WT host cells with live/dead staining, marking live bacteria in green and dead bacteria in red. The WT *S. meliloti* are healthy and differentiated. (c) Staining as in (b) of *bacA* mutant *S. meliloti* in WT host cells. The bacteria are nondifferentiated and dead except for the infection thread-located ones (arrow). (d) In an *M. truncatula dnf1* mutant nodule cell, the *S. meliloti* WT and *bacA* mutant bacteria are released from the infection threads into membrane-bound symbiosomes. The NCR peptides are not transported to the symbiosomes but remain blocked in the ER because of the absence of the DNF1 SPC subunit. Therefore, both WT and *bacA* mutant bacteria remain alive (green) within the symbiosomes but do not differentiate. (e) Staining as in (b) of WT *S. meliloti* in *dnf1* mutant host cells. The bacteria are alive but nondifferentiated. (f) Staining as in (b) of *bacA* mutant *S. meliloti* in *dnf1* mutant host cells. The bacteria are alive but nondifferentiated. Scale bars are 10 μm.

that the *bacA* gene of *Mesorhizobium huakuii* and two different *Rhizobium leguminosarum* bv. *viciae* strains were required for the formation of Fix$^+$ nodules on the legumes *Astragalus sinicus* and *Pisum sativum*, respectively (Tan et al., 2009; Karunakaran et al., 2010). Both legumes are closely related to *Medicago*, belonging to the IRLC legumes (Wojciechowski et al., 2004) and forming E-morphotype bacteroids. Histological analysis of *Pisum* nodules revealed that the *bacA*-deficient *R. leguminosarum* bv. *viciae* did not differentiate into bacteroids and could not survive in the symbiotic nodule cells but lysed after their release from the infection threads (Karunakaran et al., 2010), a phenotype similar to the *S. meliloti* mutant in *Medicago*.

In contrast, the *bacA* genes of *Mesorhizobium loti*, *Rhizobium leguminosarum* bv. *phaseoli*, *Rhizobium etli,* or

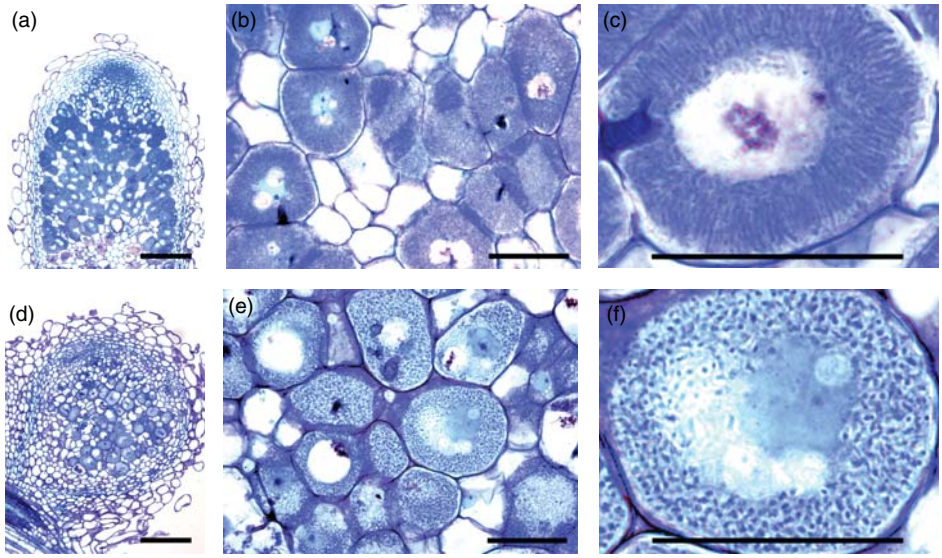

Figure 31.2 BacA is required for bacteroid differentiation in *Medicago*. Semi-thin sections stained with toluidine blue are shown of *M. truncatula* nodules infected with wild-type (WT) *S. meliloti* (a–c) or its *bacA* mutant (d–f). (a,d) Whole nodules. (b,e) Enlargement of a region containing symbiotic nodule cells. (c,f) Closeup of a single symbiotic cell, showing the elongated (E-morphotype) bacteroids in the WT infected cell (c) and the nondifferentiated bacteria in the cell infected with the *bacA* mutant (f). Scale bars are 200 µm (a,d) or 50 µm (b,c,e,f).

Sinorhizobium fredii strain NGR234 are dispensable for the formation of a functional symbiosis on various plants such as *L. japonicus*, *P. vulgaris*, *Lablab purpureus*, *Leucaena leucocephala*, *Tephrosia vogelii*, and *Vigna unguiculata* (Maruya and Saeki, 2010; Karunakaran et al., 2010; Ardissone et al., 2011). Contrary to the IRLC legumes, all these legumes form U-morphotype bacteroids and lack NCR peptides.

An eloquent example for the *bacA* requirement in symbiosis is provided by the *R. leguminosarum* bv. *viciae* strain A34 and the *R. leguminosarum* bv. *phaseoli* strain 4292, which have the same genetic background except for their symbiotic plasmids. These strains have opposing requirements for the *bacA* gene in symbiosis with *P. sativum*, which requires *bacA* and forms E-morphotype bacteroids, and with *P. vulgaris*, which does not require *bacA* and forms U-morphotype bacteroids (Karunakaran et al., 2010).

Bradyrhizobium species have a gene that is only distantly related to *bacA*, named *bclA* (see later). We have found that this *bacA*-like gene is required in *Bradyrhizobium* sp. for effective symbiosis on *Aeschynomene indica* or *Aeschynomene afraspera* whose bacteroids have the S- or E-morphotype, respectively (see Chapter 28). Mutants in the *bclA* gene do not differentiate and remain rod shaped in the symbiosomes (IG, PM, unpublished results). Strikingly, in *Bradyrhizobium japonicum*, the gene is not required for symbiosis with *G. max*, which forms U-morphotype bacteroids (IG, PM, unpublished results).

Taking together, these examples give ample evidence for a correlation between the need of the *bacA* or *bclA* gene and the formation of E- or S-morphotype bacteroids while the gene is dispensable when U-morphotype bacteroids are formed.

31.3 *BacA* PROVIDES PROTECTION AGAINST NCR PEPTIDES *in vitro* AND IN NODULES

NCR peptides induce E-morphotype bacteroid formation in IRLC legumes and unpublished evidence has demonstrated that a similar type of peptides is involved in bacteroid differentiation in *Aeschynomene* legumes. Thus the requirement of BacA or BclA proteins only for E- or S-morphotype bacteroid formation suggests that this protein plays a central role in the rhizobial response toward the NCR peptides (Kereszt et al., 2011). Two hypotheses have been put forward that could take into account the requirement of BacA (Haag et al., 2011). The protein could be directly required for the bacterial metamorphosis. However, synthetic NCR peptides induce *in vitro* bacterial elongation and DNA amplification to the same extent in the *S. meliloti bacA* mutant as in the wild-type (WT) strain, suggesting that BacA is not required for the differentiation process *per se* (Haag et al., 2011). The second possibility tested is related to the antimicrobial activity of NCR peptides. Indeed, NCR peptides resemble defensins, which are cysteine-rich AMPs of the innate immune system in animals and plants (Mergaert et al., 2003; Alunni et al., 2007). This type of immunity effector forms an early barrier against infections and has the capacity to kill invading microbes (Maróti et al., 2011). A subset of the NCR peptides possesses antimicrobial activity *in vitro* and treatment of *S. meliloti* and even other bacteria with these NCR peptides (at concentrations higher than those used for inducing bacterial differentiation) kills the microbes (Van de Velde et al., 2010; Haag et al., 2011; Tiricz et al., 2013). The killing activity of the NCR peptides is related to the damage they provoke on the bacterial membrane in a similar way as the defensins. Importantly, the *S. meliloti bacA*

mutant is between 10-fold and 100-fold more sensitive toward the antimicrobial action of NCR peptides (Haag et al., 2011; 2012). The hypersensitivity of the *bacA* mutant was not only specific to NCR peptides but applies also to the human β-defensin 2 (Arnold et al., 2013). This hypersensitivity provides a likely explanation for the mutant's symbiotic phenotype and suggests that BacA is required to survive the challenge with NCR peptides in the symbiotic nodule cells. Testing this hypothesis *in vivo*, in *M. truncatula* nodules, it was first shown that the *S. meliloti bacA* mutant bacteria, like WT, are challenged with NCR peptides once they are released from infection threads in the symbiotic nodule cells (Haag et al., 2011). With a live/dead staining procedure that marks bacteria with a different dye according to whether they are alive or dead (see Fig. 31.1), it has been found that *bacA* mutant bacteria are healthy in the infection threads but are rapidly killed after their uptake in symbiotic cells, while a WT strain remains, as expected, viable and differentiated. Moreover, the *bacA* mutant is able to survive in the symbiotic nodule cells of the *M. truncatula dnf1* mutant (Haag et al., 2011), which is affected in a nodule-specific signal peptidase complex (SPC) (Wang et al., 2010) and which cannot transport NCR peptides to the symbiosomes (Van de Velde et al., 2010). These observations are consistent with a role of BacA in preventing bacterial death by the potentially toxic NCR peptides and permitting the bacterial differentiation (Fig. 31.1). Perhaps the cell elongation and genome amplification are secondary effects of a bacterial defense response toward the antimicrobial activity of the peptides.

By synthesizing alternative versions of the NCR247 peptide with different disulfide bridge configurations between its four cysteine residues, it has been demonstrated that the peptide with disulfide bridges between cysteines 1–2 and 3–4 is significantly more active than the peptide with disulfide bridges between cysteines 1–3 and 2–4 for both induction of cell elongation and DNA amplification at low-peptide concentrations and for antimicrobial activity at higher peptide concentrations (Haag et al., 2012). Furthermore, substitution of the cysteine residues with serine diminishes the antimicrobial potency of NCR247 peptide, whereas chemically reducing the peptide by dithiothreitol treatment increases its antimicrobial potency significantly (Haag et al., 2012). This is similar to the behavior observed for the human β-defensin 1, which is produced in the colon and thought to be stored in an inactive oxidized form and to be activated by reduction through the action of thioredoxin proteins (Schroeder et al., 2011). Thioredoxins are ubiquitous redox proteins that regulate the oxidation state of cysteine residues and disulfide bridges in proteins. Intriguingly, *Medicago* nodules express a pair of unusual thioredoxins, Trx s1 and Trx s2 (Alkhalfioui et al., 2008). They are nodule specific and are thus coregulated with NCR genes. These thioredoxins differ from classical thioredoxins by the presence of an N-terminal signal peptide for the secretory pathway, such as the NCR peptides, which indicates that they are potentially cotransported with the NCR peptides to the symbiosomes. Thus, an interesting hypothesis is that these thioredoxins regulate the oxidative state of the peptides and thereby control their activity.

Importantly, no difference in sensitivity against either the chemically reduced NCR247 peptide or the linear peptide in which the cysteine residues were replaced with serine has been observed between WT *S. meliloti* and the *bacA* mutant (Haag et al., 2012). This suggests that the natural form of the NCR peptides is the oxidized one and raises the possibility that BacA can directly or indirectly influence the oxidation state of the NCR peptides in the periplasm of the bacteroids and promote the formation of the less detrimental oxidized state, thereby limiting damage of the inner membrane.

31.4 *BacA* HOMOLOGS ARE REQUIRED IN PATHOGENS FOR CHRONIC INFECTION

Apart from its role in intracellular accommodation of rhizobia during symbiosis with legume plants, BacA or BacA-like homologs have been identified and characterized in several pathogenic bacteria, in which they play critical roles during the interaction with their eukaryotic host. Notably, functional studies have been performed on BacA homologs from avian pathogenic *E. coli* (APEC) and in the mammalian pathogens *Mycobacterium tuberculosis* and *Brucella abortus*.

APEC can colonize the respiratory tract and cause pneumonia in poultry, and in addition, they are often associated with internal organ infections. In its most acute form, colibacillosis caused by APEC triggers septicemia, often resulting in sudden death of infected birds (Dziva and Stevens, 2008). As other pathogenic *E. coli*, APEC remains exclusively extracellular and the pathogen resides in the host tissues without penetrating the cells. In a signature-tagged mutagenesis screen for altered pathogenicity on chicken, a mutant in the BacA homolog (in *E. coli* named SbmA) was identified. The mutant has been shown to be attenuated in the colonization of different internal organs as compared to the WT strain, indicating that BacA is required for survival in the host and establishment of chronic infection even in the context of a nonintracellular pathogen (Li et al., 2005).

Brucella abortus is a zoonotic pathogen that causes brucellosis (Malta fever) in humans and spontaneous abortions in animal hosts such as cattle. It infects its host intracellularly through an endocytotic process and survives in phagocytic cells such as macrophages (Roop et al., 2009). A *B. abortus* BacA homolog has been identified, and the corresponding mutant displayed reduced intracellular survival

in macrophages compared to the WT strain. Moreover, bacterial clearance from infected BALB/c mice was increased in the *bacA* mutant by five orders of magnitude at 8-week postinoculation. Taken together, these results indicate that, similar to the *Rhizobium*–legume symbiosis, BacA is required for intracellular accommodation of *Brucella* and the establishment of a chronic infection (LeVier et al., 2000).

Mycobacterium tuberculosis, the causal agent of tuberculosis in humans, is a respiratory pathogen that colonizes the lung through intracellular infection. The bacterium can enter alveolar epithelial cells and macrophages and survive despite the high levels of defensins and other AMPs produced in these cells (Tan et al., 2006; Rivas-Santiago et al., 2005; Rivas-Santiago et al., 2006). In a murine infection model, *M. tuberculosis* can reside in the lung and spleen for months and lead subsequently to host death. Although the *M. tuberculosis bacA* mutant was not compromised in infection of both organs over more than 200 days, it induced significantly retarded host mortality compared to the WT strain in a long-term time-to-death experiment (Domenech et al., 2009).

Conservation of BacA function across bacterial species has been evaluated in different combinations of trans-complementation assays. Expression of the BacA homologs of either *E. coli* or *B. abortus* in the *S. meliloti bacA* mutant was able to complement its symbiotic defects and restored the formation of functional, nitrogen-fixing nodules on *M. sativa* (Ichige and Walker, 1997; Wehmeier et al., 2010). Even the *M. tuberculosis* BacA homolog rescued the *S. meliloti bacA* mutant phenotype on *M. sativa*, although only partially. In this case, nodules were not functional and did not support growth of the plants but the nodules were much more infected than in the case of the mutant, suggesting that the bacteria persisted in the nodules contrary to the *bacA* mutant, which is quickly degraded in the plant cells. Moreover, *bacA* mutant bacteria carrying the *M. tuberculosis* gene were enlarged although they displayed aberrant morphologies and did not become elongated as WT bacteroids (Arnold et al., 2013).

These examples from human and animal pathogens highlight the requirement of a functional BacA protein for inter- and intracellular accommodation of bacteria within eukaryotic cells and tissues and for maintenance of chronic infections. Similar to *S. meliloti* dealing with NCR peptides in *Medicago* nodules, these pathogens colonizing host tissues are facing the host innate immune system comprising AMPs such as defensins (Roop et al., 2009; Tan et al., 2006; Rivas-Santiago et al., 2005; Rivas-Santiago et al., 2006). It is tempting to speculate that the BacA proteins of the pathogens are important to resist to these AMPs and are therefore key in the establishment of chronic infections. In agreement with this possibility, it was shown that the *M. tuberculosis bacA* mutant has impaired tolerance to human β-defensin 2 (Arnold et al., 2013). Moreover, the *Mycobacterium* and *Brucella bacA* homologs can restore the sensitivity toward NCR peptides or human β-defensin 2 of the *S. meliloti bacA* mutant to WT levels (Haag et al., 2011; Arnold et al., 2013).

31.5 BacA IS AN ABC TRANSPORTER. BUT WHAT DOES IT TRANSPORT?

BacA and SbmA proteins have eight transmembrane domains, but they lack any other identified functional domain. However, there is a class of BacA-like proteins homologous to BacA and SbmA, which have an additional ATPase domain. The above-mentioned *Bradyrhizobium* and *Mycobacterium* genes belong to this class, and also *S. meliloti* and *E. coli* have apart from *bacA* or *sbmA* an additional *bacA*-like gene named *exsE* and *yddA*, respectively. Albeit they are divergent (LeVier and Walker, 2001), the BacA-like proteins show large regions of similarity with BacA and SbmA. In particular, transmembrane predictions revealed that all members have six transmembrane domains at conserved positions in the protein, shared with BacA and SbmA, suggesting that these proteins have similar topologies. Importantly, this class of BacA-like proteins has a fused, C-terminal ATPase domain that is typical for ATP-binding cassette (ABC) transporters (LeVier and Walker, 2001). The function of the *M. tuberculosis* BacA-like protein is abolished by deletion of its ATPase domain or by introduction of a point mutation in a conserved residue in this domain, which is known to be important for transport activity in other ABC transporters (Arnold et al., 2013). This observation suggests that BacA-like proteins indeed function as ABC proteins. Thus, potentially also the BacA/SbmA proteins are ABC transporters although their ATPase domain remains to be identified. No candidate gene has been identified in the vicinity of the *bacA*/*sbmA* genes. A recent study in *E. coli* suggested that transport by SbmA is driven by the membrane potential rather than by ATP hydrolysis, providing an explanation for the absence of an ATPase domain in SbmA (Runti et al., 2013).

Certain AMPs such as microcin B17, microcin J25, and proline-rich cathelicidin type of AMPs are internalized and have intracellular targets leading to bacterial death. These AMPs kill bacteria without affecting the bacterial membrane integrity in contrast to NCR peptides or defensins (Scocchi et al., 2011). The uptake of these peptides is mediated, at least in part, by the BacA/SbmA and BacA-like proteins, and mutations strongly reduce peptide uptake and thus confer resistance to the peptides. This has led to the suggestion that BacA/SbmA/BacA-like proteins are peptide transporters (Laviña et al., 1986; Yorgey et al., 1994; Salomon and Farias, 1995; Mattiuzzo et al., 2007; Pränting et al., 2008; Marlow et al., 2009; Karunakaran et al., 2010;

Ardissone et al., 2011; Arnold et al., 2013). Thus, the BacA/SbmA/BacA-like transporters have opposing effects on the bacterial sensitivity toward peptides with intracellular targets like the proline-rich cathelicidins and toward membrane-disrupting peptides such as NCR peptides or defensins. A possible explanation is that the internalization of AMPs by the transporter facilitates their contact with their target for AMPs with intracellular action and, on the contrary, takes them away from their membrane targets for membrane-disrupting AMPs. It is interesting to note that other ABC transporter systems are described that provide resistance to membrane-disrupting AMPs. The SapABCDF transporters in *Salmonella Typhimurium* (Para-Lopez et al., 1993; Para-Lopez et al., 1994) or the plant pathogen *Dickeya dadantii* (previously named *Erwinia chrysanthemi*) (Lopez-Solanilla et al., 1998) and the *Salmonella* YejABEF transporter (Eswarappa et al., 2008) have been proposed to import host-produced membrane-damaging AMPs taking them away from their membrane target site.

The BacA/SbmA/BacA-like proteins also mediate the uptake and sensitivity to the glycopeptide antibiotic bleomycin (Yorgey et al., 1994; Ichige and Walker, 1997; Marlow et al., 2009; Wehmeier et al., 2010; Domenech et al., 2009; Tan et al., 2009; Karunakaran et al., 2010; Maruya and Saeki, 2010) and sensitivity toward several aminoglycoside antibiotics (Ichige and Walker, 1997). Moreover, the *M. tuberculosis* BacA-like protein has recently been reported to facilitate vitamin B_{12} uptake (Gopinath et al., 2013). Although vitamin B_{12} is required for bacteroid differentiation (Campbell et al., 2006; Taga and Walker, 2010), it is unlikely that the symbiotic role of BacA or BclA is the uptake of plant-provided vitamin B_{12} because rhizobia can produce this cofactor and, more importantly, plants do not use or produce it (Roth et al., 1996).

As aminoglycoside uptake is driven by membrane potential (Taber et al., 1987) and in light of the structural diversity of all the compounds whose uptake is affected by the BacA/SbmA/BacA-like proteins, an alternative to the peptide uptake hypothesis could be that the proteins affect the membrane potential or membrane composition in a direct or indirect process. An altered membrane could have changed interaction properties with membrane-damaging peptides such as NCR peptides or defensins.

Support for a role of BacA in the determination of cell envelope features derives from the observations that the *S. meliloti* mutant has an increased sensitivity to detergents and ethanol (Ferguson et al., 2002). Similar behavior was described for *bacA* mutants in *M. huakuii* (Tan et al., 2009), *M. loti* (Maruya and Saeki, 2010), *R. leguminosarum*, or *R. etli* (Karunakaran et al., 2010). The *bacA* mutation in *S. meliloti* and *B. abortus* also reduces by about half the modification of the outer-membrane lipopolysaccharide (LPS) with typical very long-chain fatty acids (VLCFA) (Ferguson et al., 2004). Therefore, it has been proposed that BacA could be involved in the export of fatty acids out of the cytoplasm where they are synthesized (Ferguson et al., 2004). Also in *M. huakuii*, the bacA mutation affects the VLCFA content in LPS (Tan et al., 2009). However, no major changes associated with the cell envelope, including sensitivity to ethanol and detergents and lipid content of cell membranes, were detected in *bacA* or *bacA*-like deficient *Sinorhizobium fredii* strain NGR234 and *M. tuberculosis* (Domenech et al., 2009; Ardissone et al., 2011). Moreover, the symbiotic defect of the *S. meliloti bacA* mutant does not seem to be directly linked to the VLCFA LPS defect because additional mutants in the *acpXL* and *lpxXL* genes, which completely lack VLCFA in their LPS, are much less severely affected than the *bacA* mutant and can form nitrogen-fixing nodules (Ferguson et al., 2005) although bacteroid abnormalities have been observed with, for example, the formation of hypertrophied bacteroids (Haag et al., 2009).

In addition, the BacA-mediated uptake of cathelicidin type of AMPs such as the peptide Bac7 by *S. meliloti* can also be uncoupled from its symbiotic role because two point mutants in the *bacA* gene were identified that are symbiotically defective but maintain the capacity to take up the Bac7 peptide (Marlow et al., 2009). Similarly, the *M. tuberculosis bacA*-like gene can restore the resistance defect of the *S. meliloti bacA* mutant toward the NCR247 peptide as well as human β-defensin 2. Nevertheless, the *M. tuberculosis bacA*-like gene is only partially able to support symbiosis and bacteroid development (Arnold et al., 2013). Probably, the multifaceted phenotypes of the *bacA* mutant together with the complex mixture of NCR peptides produced by the symbiotic nodule cells has to be taken into account to fully explain the essential role of BacA in symbiosis.

31.6 GENERAL ENVELOPE STRESS RESPONSE FUNCTION OF BacA/SbmA?

Bacteria which interact with eukaryotic cells are often confronted with AMPs produced by the host cells. Many of these bacteria have evolved resistance mechanisms permitting them to survive these peptides. Examples are the SapABCDF and YejABEF transporters described earlier. Another frequently observed mechanism is the modification of the electrical charge of the bacterial envelope, rendering it less negative and thereby reducing electrostatic interactions with the cationic AMPs (Maróti et al., 2011). Related to this, it is interesting to note that the LPS of bacteroids in *Medicago* and pea nodules becomes more hydrophobic and displays different epitopes when compared to cultured bacteria (Kannenberg et al., 1994; Kannenberg and Carlson, 2001; Ferguson et al., 2005). This LPS modification could reflect an adaptive response of the bacteroids to the exposure with the NCR peptides.

The bacterial resistance mechanisms are usually induced after perception of the AMPs. The presence of AMPs can be directly sensed with AMP-binding two-component

regulators (Bader et al., 2005; Gryllos et al., 2008) or with ABC transporter-derived sensors, which in that case do not have a transporter function anymore but a role as receptor and signal transduction protein (Hiron et al., 2011; Falord et al., 2012). Alternatively, bacteria can sense the membrane damage provoked by the membrane-disrupting peptides through different envelope (or extracytoplasmic) stress response (ESR) systems. ESR pathways detect and induce appropriate responses to stresses that affect the bacterial membranes and periplasmic and membrane proteins, including membrane stresses provoked by AMPs (Majdalani and Gottesman, 2005; Rowley et al., 2006; Bury-Moné et al., 2009). Generally, bacteria have different coexisting ESR systems. AMP-responsive ESRs include, in *E. coli* and *S. Typhimurium*, the Rcs phosphorelay system (Farris et al., 2010), the CpxAR two-component system (Weatherspoon-Griffin et al., 2011), and the extracytoplasmic function sigma factor σ^E-regulon (Humphreys et al., 1999; Crouch et al., 2005).

Interestingly, *SbmA* is part of the ESR σ^E-regulon in *E. coli* (Rowley et al., 2006; Bury-Moné et al., 2009), suggesting that the SbmA protein has a role in ESR that needs to be further defined. σ^E and its homologs control to various degrees pathogenesis. For example, σ^E mutants of *S. Typhimurium* are defective for intracellular survival in macrophage and epithelial host cell lines and have a strongly attenuated virulence on mice (Humphreys et al., 1999; Crouch et al., 2005). The attenuated virulence of the σ^E-mutant of *S. Typhimurium* has been attributed to its inability to protect itself against the host-produced AMPs (Crouch et al., 2005). The mutant phenotypes are, in part, depending on the σ^E-regulated genes *htrA* (*degP*) and *surA*, encoding a periplasmic serine protease and a periplasmic peptidyl-prolyl-isomerase, respectively. But other σ^E-regulated genes are possibly important since σ^E mutants are more affected than individual *htrA* or *surA* mutants. A good candidate among them could be *sbmA* in light of the above-described critical function of SbmA/BacA proteins in symbiotic and pathogenic interactions.

Interestingly, a genetic link between *bacA* and *degP* was also demonstrated in *S. meliloti* (Glazebrook et al., 1996), suggesting that in *S. meliloti*, *bacA* is also implicated in ESR. However, the biochemical link between *bacA* and *degP* remains to be further unraveled.

The association of SbmA in *E. coli* and potentially also BacA in *S. meliloti* with ESR suggests that these proteins are part of a membrane stress-sensing system, or that they function in the bacterial response to alleviate this stress. The above-mentioned hypersensitivity of *bacA* mutants toward the membrane-damaging stresses induced by ethanol or detergents is in agreement with this.

The σ^E-regulon in *E. coli* includes also the *dsbC* gene (Rowley et al., 2006; Bury-Moné et al., 2009), which is involved in oxidative protein folding in the periplasm (Depuydt et al., 2011). It is unknown whether SbmA is also involved in oxidative folding, but a speculative link could be that BacA keeps NCR peptides in an oxidized and less damaging state in the periplasm as is suggested earlier (Haag et al., 2012).

ESR regulators have been described in several *Rhizobium* species (Bastiat et al., 2010; Sauviac et al., 2007; Gourion et al., 2009; Martínez-Salazar et al., 2009; see Chapter 40). But none of them were reported to regulate the *bacA* gene, and thus the regulon to which rhizobial *bacA* belongs remains to be discovered but could be very informative to understand better how BacA functions, both as a household function and in symbiosis.

31.7 CONCLUSION

BacA in *Rhizobium* species is critical for chronic intracellular infections of legume hosts, which produce AMPs in the symbiotic cells. BacA homologs are also contributing to the pathogenesis and chronic infection in pathogens, and they do this most likely by protecting the bacteria against the host AMPs. However, how BacA/SbmA proteins function mechanistically remains unknown. We propose the following hypotheses: (i) the proteins could be part of a peptide uptake system, leading to internalization of AMPs, which would move them away from membrane targets but bring them in proximity to intracellular targets; (ii) on the contrary, it cannot be excluded that they are exporters, affecting the bacterial envelop and thereby indirectly promoting the uptake of certain peptides with intracellular mode of killing and reducing the affinity of membrane-damaging peptides for the bacterial envelope; (iii) alternatively, they could export compounds that directly modifies the peptides (e.g., oxidizing peptides so that they are less harmful); or (iv) they could function as sensors rather than transporters, activating other resistance determinants. Testing these possibilities is a challenge for the future. Understanding the household function of *bacA* in *Rhizobium* and its regulation in stress response or other cellular functions could be an important clue for understanding its interference with the action of NCR peptides and other AMPs.

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Chapter 32

Molecular Keys to Broad Host Range in *Sinorhizobium fredii* NGR234, USDA257, and HH103

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32.1 INTRODUCTION

Gram-negative soil bacteria that form a symbiotic relationship with legumes are collectively referred to as rhizobia. They are classified as Alphaproteobacteria and Betaproteobacteria (Moulin et al., 2001; Peter et al., 1996; Sadowsky et al., 2013; Skerman et al., 1980) (Fig. 32.1).

Their nitrogen-fixing capacities are of tremendous environmental importance to the global nitrogen cycling balance (Cheng, 2008) and the agricultural economy (Sessitsch et al., 2002). The rhizobia–legume symbiosis is initiated by a dialog between the legume plant and the compatible *Rhizobium* in the soil (Deakin and Broughton, 2009; Gage, 2004; Jones et al., 2007). Plants liberate flavonoids and also other compounds (such as B vitamins) into the rhizosphere. These signaling molecules upregulate rhizobial *nod* genes, subsequently lipochitooligosaccharidic nodulation factors (Nod factors) are produced that trigger the nodulation pathway in susceptible legumes (Oldroyd et al., 2011; see Chapters 50, 51). As a result, rhizobia enter the plant root through root hairs, make their way to the cortex, multiply, and initiate the formation of specialized root structures called nodules facilitating the access to mineral nitrogen via the root (Gage, 2004). Although some rhizobia are able to reduce atmospheric nitrogen to ammonia under free living conditions, the reduced oxygen tension found within root nodules maximizes the process of nitrogen fixation (Downie, 2005). Interestingly, the specificity of this interaction is determined by a number of molecular keys that are linked to the synthesis of Nod factors (i.e., lipochitooligosaccharides and their decorations), the receptors of plant signaling molecules (i.e., NodD proteins), and the arsenal of secretory components encoded by the various rhizobia (Table 32.1).

These are perhaps major determinants of host range (Berck et al., 1999; Broughton et al., 2000). However, it can be assumed that host range is also a result of rhizosphere fitness and a mix of other genetically encoded determinants that are not fully understood yet and only will be unraveled once we have a broader understanding of rhizobial "omics."

It is perhaps noteworthy that many rhizobia have evolved mechanisms that allow them to nodulate a larger variety of legume plants. These strains are called broad host range strains and are promiscuous with respect to the selection of their host plants. A detailed overview on the best studied model organism is given in the reviews (Berck et al., 1999; Marie et al., 2001). While the basic principles that are linked to host range determination are only now falling into place, the observation of this phenomenon has been made over a hundred years ago (Hiltner and Störmer, 1903; Wilson, 1939). While Hiltner and his team were perhaps the first to report on host range restrictions, Wilson and his team carefully analyzed the host range properties of rhizobia, which they had isolated from 31 different legumes genera and from 160 different legume species.

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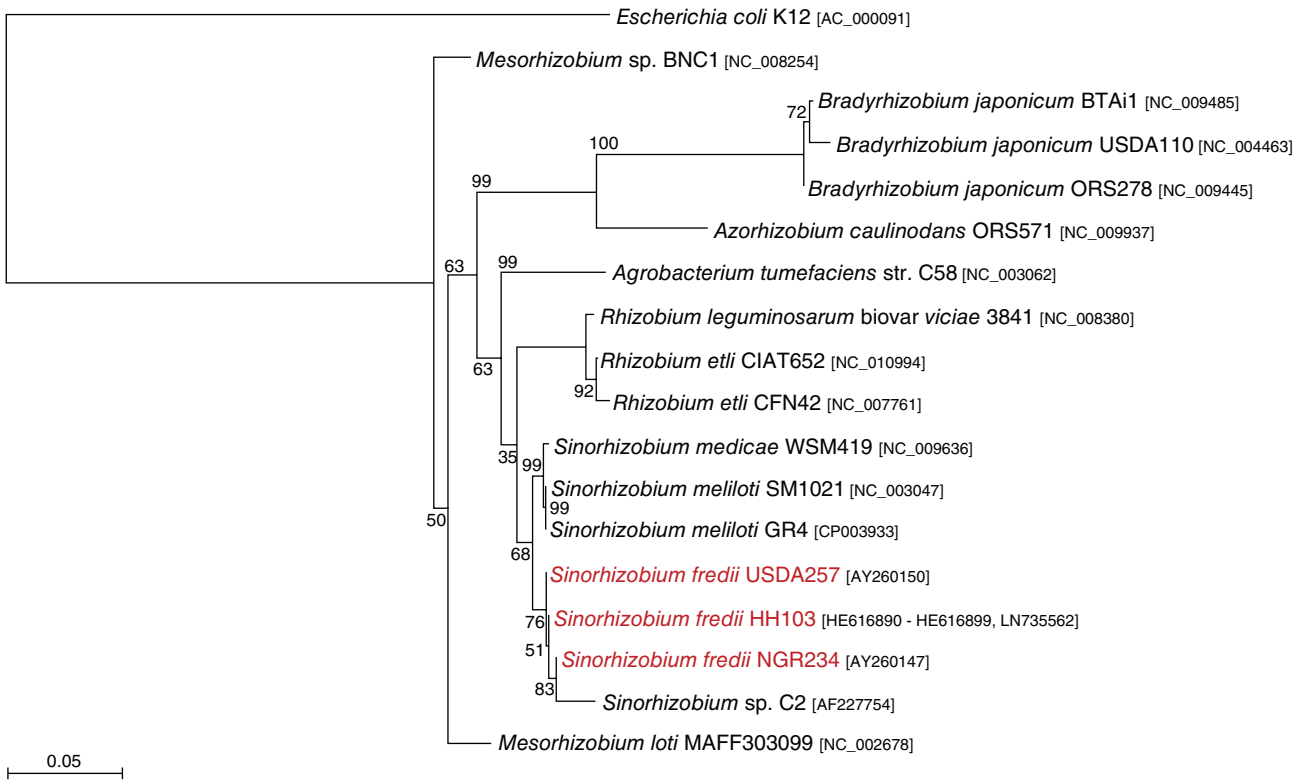

Figure 32.1 Phylogenetic relationships of 16S rRNA genes from selected rhizobial species as determined by the maximum-likelihood analysis. The percentage of bootstrap resamplings is indicated at the different nodes. The scale bar represents the expected number of changes per nucleotide position.

They clearly demonstrated that all the obtained isolates nodulated legumes of different tribes. The average number of plant species nodulated by a particular strain was 33%, clearly suggesting that rhizobial promiscuity is perhaps widely distributed in nature, and that this phenomenon is more the rule than the exception (Wilson, 1939).

Within this framework, currently three *Sinorhizobium fredii* isolates are studied that are known broad host strains (Fig. 32.1). These are the closely related *S. fredii* isolates NGR234, USDA257, and HH103 (Marie et al., 2001). However, the latter isolate shows a rather narrow host range. In the following section, key traits of these different *S. fredii* isolates are highlighted.

32.2 KEY TRAITS OF THE ALPHAPROTEOBACTERIA *Sinorhizobium fredii* NGR234, USDA257, AND HH103

32.2.1 *Sinorhizobium fredii* NGR234

Within the large group of the rhizobia, NGR234 (Fig. 32.1) portrays an outstanding candidate forming nitrogen-fixing root nodules with over 110 genera of legumes as well as with the nonlegume *Parasponia andersonii* (Pueppke and Broughton, 1999). NGR234 was first uncovered in 1965 in Papua New Guinea and isolated from *Lablab purpureus* nodules as the only fast-growing strain among 30 isolates (Trinick, 1980). Shortly after this discovery, its broad host range attracted great interest since no other plant symbiont was discovered having as many symbiotic partners as NGR234. Nevertheless, the molecular mechanisms behind this exceptionally wide host range are not fully evident, therefore qualifying NGR234 as the ideal prokaryotic model organism for studies with relevance to symbiotic determinants (Broughton et al., 2000).

Early studies with derivates and spontaneous resistance mutants of NGR234 yielded evidence that genes required for an effective symbiosis with leguminous plants are located exclusively on the symbiotic plasmid (pNGR234*a*) (Freiberg et al., 1996; Freiberg et al., 1997; Perret et al., 1991). A NGR234 derivate (ANU265) that was deprived of pNGR234*a* by heat curing was not impaired in its growth but failed to nodulate any of its hosts (Morrison et al., 1983). Consequently, the 0.5 Mb symbiotic plasmid of NGR234 lacks essential genes required for growth and survival control but comprises symbiotic determinants, for example, genes for nodulation (*nod*) and synthesis of Nod factors as well as genes for nitrogen fixation (*nif/fix*) (Freiberg et al., 1997).

Table 32.1 Identified secretion systems and assumed bacterial determinants of host range in the *Rhizobium*–legume symbiosis

Determinant	Identified as a Determinant of Host Specificity?	Type/Origin	Examples/Structure	Mode of Action	Source
Flavonoids	Yes/no	Plant signals (inducers or inhibitors)	e.g., Flavone, flavonole, flavanone, isoflavonoids and nonflavonoid substances	Flavonoids induce *nodD* expression and interact with NodD proteins building up a complex	Perret et al. (2000); Hirsch et al. (2001)
NodD protein	Yes	Rhizobial sensor of plant signals and transcriptional activator	LysR-like transcriptional regulator	NodD protein binds to *nod* boxes and regulates transcription of nodulation genes (*nod*, *nol*, *noe*), which are subsequently involved in synthesis of Nod factors	Schlaman et al. (1992); Fisher et al. (1993)
Nod factors	Yes	Rhizobial signal molecules	Lipochitooligosaccharide backbone with different decorations	Nod factors are recognized by the plant and induce early nodule development/rhizobial infection	Relic et al. (1993, 1994)
NodO protein	Yes	Rhizobial secreted protein	Calcium-binding protein	NodD-dependent expression of NodO, it can either facilitate Nod factor uptake, amplify the perceived Nod factor, or bypass the Nod receptor	de Maagd et al. (1989); Walker and Downie (2000)
Surface polysaccharides	Yes	Rhizobial surface compounds	EPS, LPS, KPS	Surface polysaccharides are involved in early stages of the infection processes (plant root invasion/nodulation) and facilitate the infection thread penetration	Fraysse et al. (2003); Downie (2010); Skorupa et al. (2006)
T3SS and effector proteins	Yes	Export of rhizobial effector proteins	e.g., NopL, NopP	T3SSs control the translocation of effector proteins that affect nodule initiation, these can, e.g., block plant defense mechanisms or induce certain symbiotic actions	Viprey et al. (1998); Deakin and Broghton (2009); Perret et al. (2000)
Protein secretion systems	?	Export of multifaceted proteins	T1SS–T6SS	Secretion systems translocate different classes of rhizobial proteins into the surrounding environment or into the eukaryotic host	Fauvart and Michiels (2008); Krehenbrink and Downie (2008)
Cell–cell communication	?	QS: different signaling molecules	QS: Autoinducer, e.g., *N*-AHLs	*N*-AHL-mediated signaling for coordinated expression of genes for successful infection	Zhang et al., 1993; Brelles-Marino and Bedmar (2001); Gonzalez and Marketon (2003)
		QQ: rhizobial (secreted) proteins	QQ: AHLases, amidases, oxidoreductases, hydrolases	QQ-based blockade of QS in other microorganisms for competition purposes	Uroz et al. (2005); Dong et al. (2000)

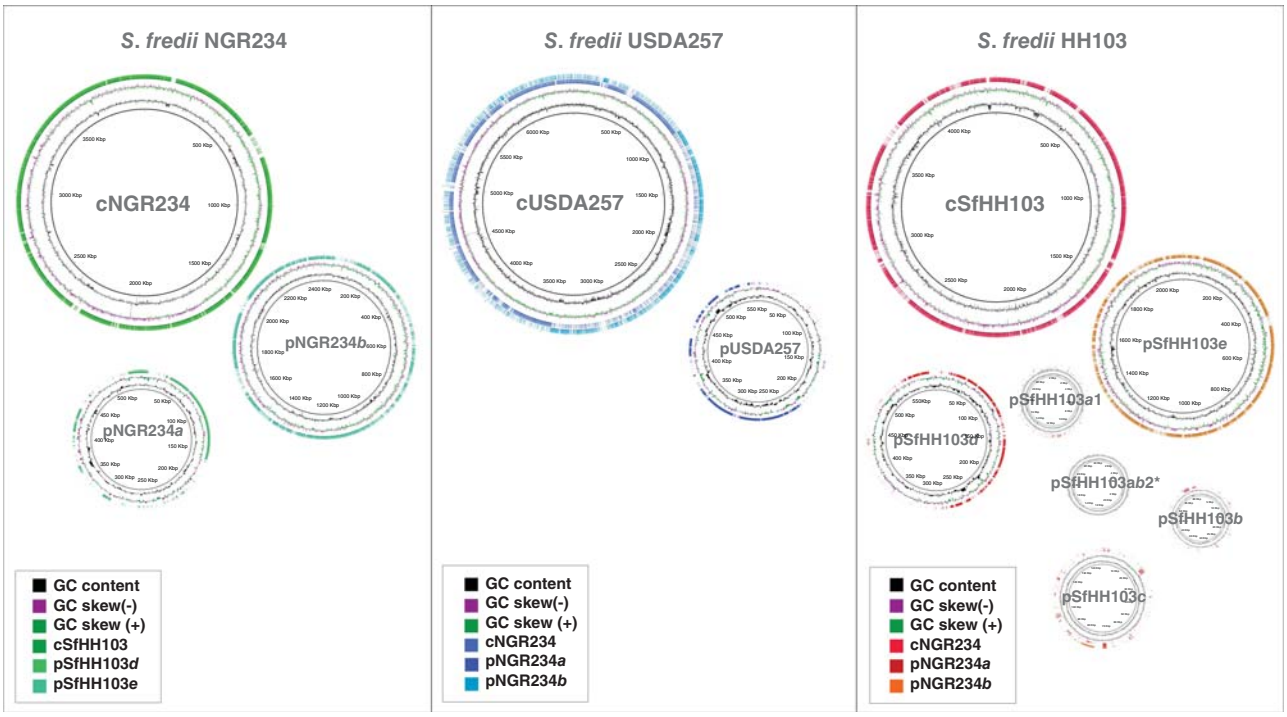

Figure 32.2 Genome maps and BlastN comparisons of NGR234, USDA257, and HH103. The innermost rings indicate the GC content (black) and GC skew (purple/green). The outermost rings represent the shared identity to the best matching replicon of the other *S. fredii* strain; the homologous regions between the two replicons are depicted in color. Shared identities in NGR234: the chromosome cNGR234 has the highest similarity to the chromosome cSfHH103, the sym plasmid pNGR234*a* has the highest similarity to pSfHH103*d*, the megaplasmid pNGR234*b* has the highest similarity to pSfHH103*e*; shared identities in USDA257: the chromosome cUSDA257 shares the highest similarity to chromosome cNGR234 and megaplasmid pNGR234*b*, the plasmid pUSDA257 has the highest similarity to pNGR234*a*; shared identities in HH103: the chromosome cSfHH103 has the highest similarity to chromosome cNGR234, the small plasmids pSfHH103*a*1-*e* share only weak similarities to NGR234's genome (cNGR234/pNGR234*a*/pNGR234*b*), the sym plasmid pSfHH103*d* has the highest similarity to sym plasmid pNGR234*a*, the megaplasmid pSfHH103*e* has the highest similarity to megaplasmid pNGR234*b*. *Plasmid pSfHH103*a*2 was not considered in the comparisons. Genome sequences were derived from the corresponding GenBank entries, genome maps were created using BRIG (Blast Ring Image Generator; http://sourceforge.net/projects/brig).

In a symbiotic relationship, root hair curling, induction of nodulation, and the entry of bacteria into the root are strictly dependent on the secreted Nod factors (see Chapters 51, 59, 63), thus they play a key role in the host range of symbiotic soil bacteria such as NGR234 (Viprey et al., 2000).

Most intriguing was the discovery of the first type III secretion system (T3SS) in a rhizobial genome by the work of Freiberg and colleagues. This was uncovered during the sequencing of the pNGR234*a* replicon, which was the first rhizobial replicon to be sequenced in 1997 (Freiberg et al., 1997). Later during the course of sequencing the whole 6.9 Mb NGR234 genome, a second copy of a T3SS was identified (Schmeisser et al., 2009). However, the molecular function of this second T3SS with relevance to root colonization, infection, and nodule formation is not yet clear. The NGR234 genome is divided into three replicons, a 0.5 Mb symbiotic plasmid (pNGR234*a*), a 2.4 Mb megaplasmid (pNGR234*b*), and the bacterial chromosome (cNGR234) with a size of 3.9 Mb (Fig. 32.2).

Among many other striking features, NGR234 encodes for a wealth of secretion-related proteins, which play an important role in the infection of plant hosts (Deakin and Broughton, 2009; Schmeisser et al., 2009). The genome revealed the presence of many proteins involved in type I (T1SS) and type II secretion (T2SS); apart from the above-mentioned T3SS, two complete conjugative type IV secretion systems (T4SS) were observed. No type V or VI transporters (T5SS/T6SS) were found within this genome (Table 32.2). Altogether, a relatively high number of 129 genes, which are spread over the three replicons of NGR234, are in some form involved in secretory processes in this microbe (Schmeisser et al., 2009).

Rhizobial cells have to be present in large numbers in and around plant root hairs as well as in nodules in order to achieve a successful symbiotic interaction (Bauer and Mathesius, 2004). To monitor their own population in the rhizosphere and to subsequently increase the cell density, cell-to-cell signaling processes are needed. Among

Table 32.2 Putative secretion-associated genes and proteins identified in *S. fredii* NGR234, USDA257, and HH103 (Data derived from Schmeisser et al., 2009; modified; Schuldes et al., 2012; Weidner et al., 2012).

		S. fredii NGR234				*S. fredii* USDA257			*S. fredii* HH103				
Secretion System		cNGR 234	pNGR 234*a*	pNGR 234*b*	**Total**	cUSDA 257	pUSDA 257	**Total**	cSfH H103	pSfHH 103*a–c*	pSfHH 103*d*	pSfHH 103*e*	**Total**
Type I		4		2	**6**	8	1	**9**	2		2	2	**6**
Type II		13			**13**				10				**10**
Type III			20	22	**42**	21	22	**43**			20	11	**31**
Type IV	F-type		12	11	**23**	13	24	**37**			30	13	**43**
	P-type		12	23	**35**	33		**33**	12			24	**36**
Type V													
Type VI						22		**22**					
TAT		3			**3**	3		**3**	3				**3**
Sec		7			**7**	7		**7**	8				**8**
					129			**154**					**137**

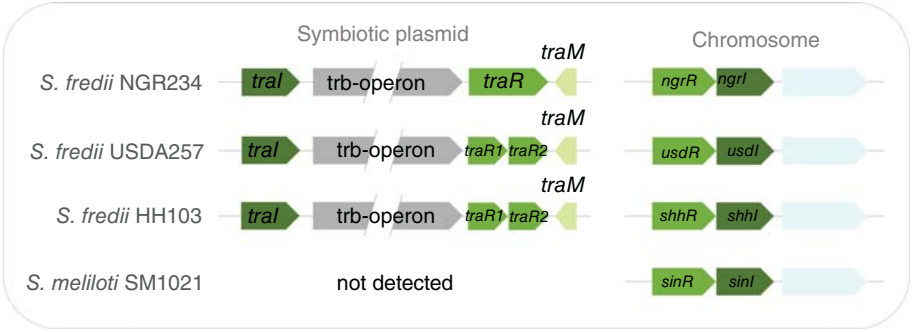

Figure 32.3 Cell–cell communication systems identified in the genomes of broad host strains *S. fredii* NGR234, USDA257, and HH103 and the narrow host strain *S. meliloti* SM1021. Left: Conventional QS system responsible for synthesis of AHLs is found on the symbiotic plasmid of NGR234, USDA257, and HH103. USDA257's putative regulators traR1 and traR2 have not yet been functionally confirmed. The narrow host range strain *S. meliloti* SM1021 lacks *traI/traR* homologs. In all three strains, the AHL synthase TraI directs the synthesis of AHL, which associates with the response regulator TraR. TraM functions as a suppressor, preventing TraR from activating target genes under noninducing conditions. The flanked *trb*-conjugal operon is shaded gray. Right: Second, novel QS system identified on the chromosome of all four rhizobial strains. It is composed of the autoinducer synthase gene (*ngrI*, *usdI*, *shhI*, and *sinI*) and the response regulator (*ngrR*, *usdR*, *shhR*, and *sinR*) in an inverted order and a hypothetical protein (blue) of unknown function. This system is most likely producing a coumestrol autoinducer type I signal.

Rhizobiaceae, diverse quorum sensing (QS) systems are prevalently used to exchange signaling molecules such as *N*-acyl homoserine lactones (*N*-AHLs) (Loh et al., 2002; Wisniewski-Dye and Downie, 2002; see Chapter 37). With respect to cell–cell communication, NGR234 carries two QS systems that are responsible for the autoinducer synthesis and recognition (Fig. 32.3). In 2003, He and colleagues already described the NGR234 QS regulators TraI and TraR producing and operating with 3-oxooctanoyl-homoserine lactone (He et al., 2003). This TraI/TraR system together with its suppressor TraM flanks the conjugal transfer operon and can be found on pNGR234*a*. A second putative QS system composed of NgrI/NgrR was identified being located on NGR234's chromosome most likely producing coumestrol autoinducer I type molecules (Krysciak et al., 2011). A recent genome wide RNA-seq analysis of NGR234-ΔtraI and NGR234-ΔngrI mutants identified major traits in NGR234 that were controlled by the autoinducer synthases TraI and NgrI. Beside many genes and operons regulated either by TraI/R or NgrI/R, a common set of 186 genes was differentially regulated by both QS regulons including flagellar biosynthesis genes and genes linked to exopolysaccharide biosynthesis (Krysciak et al., 2014).

Regarding the multiple proteins and gene operons participating in QS in NGR234, this microbe also developed quorum quenching (QQ) strategies to disarm QS molecules in order to compete with other bacterial species in nutrient and energy-limited niches (Teplitski et al., 2011). Using a genome-wide functional analysis conducted in 2011 in our

laboratory, we could demonstrate that NGR234 carries a surprisingly large number of genes encoding enzymes involved in the degradation or modification of *N*-AHLs. NGR234's most promising QQ-associated proteins DlhR and QsdR1 exhibited a clear QQ activity in all biosensor tests. Furthermore, these proteins contributed to the rhizosphere fitness of NGR234 and are possibly involved in the degradation of plant-derived molecules in the rhizosphere, emphasizing the ecological importance of QQ during root colonization (Krysciak et al., 2011).

32.2.2 *Sinorhizobium fredii* USDA257

The phylogenetically closely related strain USDA257 (Fig. 32.1) was isolated from a soybean plant (*Glycine soja*) growing in China (Keyser et al., 1982) and is perhaps the best studied broad host model organism nodulating soybeans effectively. This fast-growing isolate, which was generally considered to be a soybean symbiont, nodulates an exact subset of 79 genera of NGR234 plant hosts. Consequently, USDA257 was classified as a member of the broad host range rhizobia (Pueppke and Broughton, 1999).

The complete genome sequence of USDA257 revealed the presence of only two replicons: the chromosome of USDA257 (cUSDA257) and the plasmid (pUSDA257) in total 7.03 Mb (Schuldes et al., 2012) (Fig. 32.2). Similar to NGR234's symbiotic plasmid, pUSDA257 lacks essential genes for the control of survival and growth but encodes many genes linked to symbiosis (*nod*/*nif*/*fix*). In addition to the plasmid-borne *nif* and *fix* genes, a significant number of *nif* and *fix* genes are located on the chromosome of USDA257. Furthermore, USDA257 is perhaps able to grow on a wide range of substrates, including aromatic compounds (Schuldes et al., 2012). Similar to NGR234, the genome of USDA257 encodes for a surprisingly high number of secretion-associated genes and proteins. In total 154 genes could be identified being putatively linked to secretory processes (Table 32.2). Unlike NGR234, USDA257 does not appear to encode a functional T2SS but in return comprises a cluster of T6SS-related genes, which is very unique and has been found only in a few rhizobial species such as *R. leguminosarum*, *B. japonicum*, and *Mesorhizobium loti* (Records, 2011). Furthermore, the genome of USDA257 harbors two complete sets of T3SSs, one located on the chromosome and one on the symbiotic plasmid. This unique feature makes USDA257 and NGR234 to our knowledge the only known rhizobial strains with two copies of T3SSs (Table 32.2) (Schuldes et al., 2012).

A highlight of the USDA257 genome is also the presence of many QS and QQ related genes (see Chapter 37). Similar to NGR234, the USDA257 genome encodes for two QS systems, most likely involved in the synthesis of *N*-AHL autoinducer-like molecules. The autoinducer synthase genes and their respective regulators were designated *traI*/*traR1; traR2* and *usdI*/*usdR* (Fig. 32.3). However, the functions of USDA257's *traR1* and *traR2* putative response regulators have not yet been experimentally confirmed. In addition and similar to NGR234, USDA257 carries a significant number of loci linked to modification or degradation of autoinducer molecules (Schuldes et al., 2012).

32.2.3 *Sinorhizobium fredii* HH103

Apart from the two above-described broad host isolates, the soybean-nodulating isolate HH103 has been the focus of many studies. The fast-growing rhizobial strain *S. fredii* HH103, which was originally isolated from Chinese soil from the Hubei Province in 1985, is able to form nodules on a limited number of legume cultivars (Dowdle and Bohlool, 1985). A more recent analysis suggested that about 15 legume genera including advanced soybean variants are susceptible to infection through HH103 and result in nitrogen-fixing nodules (Margaret et al., 2011). While HH103 is promiscuous, its host range clearly differs from the host ranges of NGR234 and USDA257 and is less wide. Competitive nodulation studies revealed that *Bradyrhizobium japonicum* strain USDA110 outcompetes HH103 in an acid soil, whereas in more alkaline soils the majority of the nodules are colonized by HH103 (Buendía-Clavería et al., 1994). The HH103 genome has been partially established recently (Weidner et al., 2012; Vinardell et al., 2015). Surprisingly, the 7.25 Mb genome of HH103 is spread over six replicons (pSfHH103*a*1/2–*e*) and a circular chromosome of 4.3 Mb. The six smaller circular plasmids range from 24 kb up to 2 Mbs in size (Margaret et al., 2011; Vinardell et al., 2015) (Fig. 32.2).

The partial HH103 genome shares a high degree of synteny with the NGR234 and USDA257 genomes. Similar to these two microbes, the symbiotic plasmid (pSfHH103*d*) of HH103 carries genes-encoding proteins relevant to nodulation and nitrogen fixation and additionally encodes a single T3SS (Weidner et al., 2012). The largest plasmid, pSfHH103*e*, carries genes involved in the production of surface polysaccharides such as exopolysaccharides (EPSs), lipopolysaccharides (LPSs), and capsular polysaccharides (KPSs), and the smaller plasmids pSfHH103*a*1/2 and pSfHH103*b* reveal the presence of numerous transposases (Margaret et al., 2011; Weidner et al., 2012; Vinardell et al., 2015).

Our first attempts to describe the secretion machinery composed of HH103 revealed the presence of around 137 secretory genes and proteins only spread on the chromosome, symbiotic plasmid, and the largest plasmid. Although the wealth of secretion-associated genes is very similar to NGR234, HH103 lacks the second complete copy of the T3SS. In contrast, HH103 carries almost twice as many

T4SS-related proteins (F-type) than recorded for NGR234. Similarities to T5SS and T6SS-associated proteins were not recorded (Table 32.2). Surprisingly, none of the identified secretion-related proteins were located on the four smaller plasmids pSfHH103*a*1/2, pSfHH103*b*, or pSfHH103*c*.

With respect to cell–cell communication, HH103 seems to be similarly equipped with QS and QQ-related genes as NGR234 and USDA257 (see Chapter 37). First alignment studies with HH103 and already published QS systems found among the Rhizobiaceae show that HH103 possesses also two systems. Clear homologs, which are to date annotated as hypothetical proteins in HH103, could be found to the QS *traI/traR* system originally described in *A. tumefaciens*. Similar to NGR234 and USDA257, this system is located on the symbiotic plasmid (pSfHH103*d*) of HH103 flanking the putative conjugal transfer operon (Fig. 32.3) and is probably responsible for the synthesis and recognition of *N*-AHLs. The second system with the proposed name *shhI/shhR* is located on the chromosome of HH103 and also shows an inverted order of synthase and regulator genes compared to *traI/traR*. Based on the similarity to the *ngrI/ngrR* and *usdI/usdR* system, *shhI* is most likely producing a coumestrol-like autoinducer.

To date, no experimental evidence exists that HH103 is able to exhibit QQ as a defense strategy in environments like the soil. Nevertheless, first comparative studies show that this rhizobial strain carries analogs of NGR234's QQ-related proteins, which are responsible for modification or degradation of AHLs.

32.3 MOLECULAR KEYS TO BROAD HOST RANGE IN *S. fredii* NGR234, USDA257, AND HH013

Broad host range is probably a result of many features that are encoded within the different rhizobial genomes (Table 32.1). Although the complete genome sequence of NGR234, USDA257, and HH103 has not revealed all of the mysteries of host specificity, probably many more pieces of the broad host range puzzle will fall into place once more "omic"-based studies are performed by fully exploiting the available genome sequences.

32.3.1 Protein Secretion as a Key to Host Range

One important observation is that correlations seem to exist between the host range of rhizobia and the number and type of specialized protein secretion systems they carry. Classic narrow host range rhizobia such as *S. meliloti* and *R. leguminosarum* that only nodulate a very restricted group of Middle Eastern and Northern European plants lack T3SSs and T4SSs, respectively (Galibert et al., 2001; Krehenbrink and Downie, 2008). NGR234, however, which has the greatest capacity to nodulate of all known rhizobia, carries almost twice as many, often duplicated, secretion systems then these narrow host model microbes. USDA257 and HH103 also both encode for more than 130 genes linked to the setup of secretory machines (Table 32.2). While USDA257 obviously lacks a functional T2SS it encodes a T6SS, which is absent in NGR234 and HH103. Specifically, the importance of the T3SS and the array of secreted nodulation outer proteins (Nops) have been elucidated in recent years in many studies for NGR234, USDA257, and HH103, clearly providing convincing evidence that a functional T3SS and secreted Nop effector proteins are keys to broad host range. Therefore, several mutational analyses were accomplished in NGR234, USDA257, and HH103 focusing on different components of the T3SS. The inactivation of selected T3SS-related genes in these three rhizobial strains abolished the secretion of various flavonoid-induced Nop effector proteins (e.g., NolX and NopL) and furthermore decreased their symbiotic capacity (de Lyra et al., 2006; Krishnan, 2002; Krishnan et al., 2003; López-Baena et al., 2008; Viprey et al., 1998; Zhang et al., 2011). Moreover, these studies suggested that Nops can act either as beneficial or detrimental symbiotic factors in a host-dependent manner (de Lyra et al., 2006).

Since only two of the *S. fredii* broad host range strains appear to encode for two copies of a T3SS, future work will have to elucidate the role of the second T3SS in these particular microbes. Furthermore, formal proof that any of the other diverse protein secretion systems identified in these microbes extending the host range of NGR234 has yet to be furnished.

32.3.2 NodD Proteins as First Modulators of Host Specificity

Rhizobial cells recognize secreted plant signals (flavonoids or isoflavonoids) by a sensor protein and LysR-type transcriptional activator usually encoded by *nodD* (Fisher and Long, 1993; see Chapter 51). The activated NodD protein binds to highly conserved DNA motifs of the so-called nodulation boxes (*nod boxes*), which are found in promoter regions of many nodulation loci. After binding, NodD regulates transcription of several nodulation genes (*nod/nol/noe*) (Gyorgypal et al., 1991; Schlaman et al., 1992). To recognize the different plant signals rhizobia have often multiple NodD homologs, which can vary in their preferences for flavonoids (see Chapter 50). Additionally, the symbiotic characteristics of NodD proteins can vary between different rhizobial species (Broughton et al., 2000). NGR234's NodD1 protein usually responds to a wide palette of inducing compounds excreted by the host plant. Transferring the *nodD1* gene of NGR234 into narrow host range strains such as *S. meliloti* and *R. leguminosarum* extended their nodulation capacity and thereby their host range. Nevertheless, NodD1 isolated

from these narrow host range isolates could not restore the ability of an NGR234-*nodD1* mutant to nodulate some of its original legume hosts (Relic et al., 1994; Spaink et al., 1987). In fact, these experiments show that some correlations can be made between the flavonoidic interaction with the different NodD proteins and the host specificity.

32.3.3 Nod Factors Are Keys to Broad Host Range

Binding of the NodD protein to *nod boxes* initiates the expression of nodulation genes, which are subsequently involved in the synthesis and secretion of Nod factors. These structures are oligomers of four or five 1,4-linked *N*-acetyl-glucosamine residues with different substitutions that are attached to the backbone (Spaink, 2000; see Chapter 51). The assemblage of the substitution and the backbone results in a highly specific Nod factor that is recognized by a particular legume host plant, allowing only certain rhizobia–plant associations to occur. Consequently, host specificity in these symbiotic interactions can be determined by the various Nod factors that are generated by different species of rhizobia (Downie, 1998).

The broad host range strain NGR234 secretes a large family of Nod factors that are 3-*O*, 4-*O*, or 6-*O* carbamoylated, *N*-methylated, and carrying a 2-*O*-methyl-fucose residue that may be either 3-*O* sulfated or 4-*O* acetylated. Currently, no other rhizobial isolate is known to synthesize such a wide range of lipochitooligosaccharides (Relic et al., 1993; Relic et al., 1994). Yet another piece of evidence suggests that Nod factors are keys to host range. Not only NGR234 treats the legume root with a large palette of Nod factors but also their concentration is much higher compared to Nod factors produced by very closely related rhizobia (Relic et al., 1994). Thus, it is reasonable to speculate that Nod factors and especially their numerous decorations are important keys to host range determination (Broughton et al., 2000; Deakin and Broughton, 2009; Perret et al., 2000). And although these factors are essential for nodulation, these signal molecules seem to be probably just one of several elements specifying host range.

32.3.4 NodO – a NodD Regulated Protein

One of the rhizobial nodulation proteins that are expressed under the control of the *nodD* gene product is NodO. This secreted protein contains a calcium-binding site and is proposed to be translocated by the T1SS (Sutton et al., 1994). Its role in symbiosis was reported in 1998, where a *Rhizobium* sp. BR816 *nodO* mutant was found to be impaired in its ability to nodulate a certain host strain (Vlassak et al., 1998). Furthermore, the *nodO* gene from *Rhizobium* sp. BR816 and also the *nodO* gene from *R. leguminosarum* bv. *viciae* could extend the host range of narrow strains such as *S. meliloti* (Economou et al., 1990). Additionally, in the absence of host-specific *nod* genes, NodO can compensate for the loss of the appropriate Nod factor. Although the role of NodO is still unclear, three functions could be assigned: NodO facilitates the uptake of the Nod factor, it can amplify the Nod factor; or it can bypass the host's Nod factor receptor (Walker and Downie, 2000).

Surprisingly, only very low sequence similarities to NodO could be found with the genomes of NGR234, USDA257, and HH103. Nevertheless, the previously mentioned results suggest that NodO plays an essential role in symbiosis in *R. leguminosarum* bv. *viciae* and few other rhizobial candidates (de Maagd et al., 1989).

32.3.5 Metabolic Capabilities

The diverse catabolic and metabolic functions encoded by NGR234, USDA257, and HH103 are perhaps another piece of the host range puzzle. Rhizobia have at least two lifestyles: one saprophytic and the other symbiotic and intracellular. A soil bacterium that is able to survive and grow under a wide range of nutritional conditions has more chances of surviving than strains that have very specific growth requirements (Oldroyd et al., 2011). More importantly perhaps, to be primed for the invasion of legumes rhizobia have to grow in the rhizosphere of approaching root systems. Bacteria that can metabolize virtually any carbon- and nitrogen-containing compound that emanates from plant roots will preferentially colonize them (Poole and Allaway, 2000; Sadowsky et al., 2013). This would also include the modification or perhaps degradation of the many flavonoid-signaling molecules released by plants as a primary defense barrier against microbial colonization. However, no data are available to support this hypothesis.

32.3.6 Surface Polysaccharides are Essential for Symbiosis

To establish a successful rhizobial–legume symbiosis, bacteria have to adapt to changing environmental conditions that occur in the proximity of plant roots. The bacterial surface is the first line of defense and in the plant–microbe interaction the first phase of attachment to root hairs. In response to the environment, rhizobia modify their outer cell surface composed of a number of different polysaccharides such as EPSs, LPSs, and KPSs (Fraysse et al., 2003; Skorupska et al., 2006). Although the exact molecular function of these surface polysaccharides remains unclear, their role in establishing a symbiosis with legumes has been reported. EPSs appear to prevent plant defense responses and thus are involved in early infection processes (Parniske et al., 1994; Staehelin et al., 2005; see Chapter 36). In contrast, LPSs play a role in the later stages of the nodulation processes (Carlson

et al., 2010). KPSs are tightly associated with the rhizobial outer membrane, are thus distinct from the loosely adhering EPS, and seem to promote the initiation and development of the infection thread (Le Quere et al., 2006). Rhizobial KPS are strain-specific antigens that are structurally analogous to the group II K-antigens of *E. coli* (Kannenberg et al., 1998).

Detailed genomic analyses proved that most genes linked to surface polysaccharide synthesis are located on the megaplasmid of NGR234. More than 25 genes stretching from *exoU* (NGR_b19500) to *exsI* (NGR_b01520) are involved in the synthesis of low-molecular-weight EPSs that are essential for nodule invasion on some plants (Staehelin et al., 2006). Furthermore, three clusters of genes (*rkp-1*, *rkp-2*, and *rkp-3*) are involved in the production of the K_R5 antigen in NGR234 (Becker et al., 2005). Chromosomally located *rkp-1* and *rkp-2* clusters are probably responsible for the production of a specific lipid carrier necessary for the synthesis of KPS and in the metabolism of nucleotide diphosphosugars, respectively (Schmeisser et al., 2009). In contrast, *rkp-3* is carried by pNGR234*b*.

32.3.7 Cell–Cell Communication Mechanisms as Novel Keys to Host Range

N-AHL-mediated signaling enables rhizobial strains to sense their local environment (rhizosphere) to subsequently coordinate the expression of certain genes. Such a synchronized gene expression is of great importance for bacterial symbionts as a successful infection strategy of eukaryotic hosts, as well as the ability to establish an effective symbiosis, requires apart from other signaling pathways a QS-dependent signaling (Bauer and Mathesius, 2004; LaSarre and Federle, 2013; Wisniewski-Dye and Downie, 2002). Most QS circuits found in plant-associated bacteria are similar in structure; however, the subordinated genes mediating physiological processes are diverse. The underlying QS signaling systems are often complex owing to the incorporation of multiple AHL synthase and response proteins and their organization in a regulatory hierarchy (Soto et al., 2006). Rhizobial QS regulatory systems that have a direct effect on symbiosis have already been described in *R. leguminosarum* bv. *viciae* (Cubo et al., 1992), *R. etli* (Daniels et al., 2002), and *S. meliloti* (Gurich and Gonzalez, 2009; see Chapter 37). The studies mainly working with mutations of single QS-related genes demonstrated the importance of QS for nodulation and nitrogen fixation. These studies could, in part, verify that QS systems regulate diverse genes that are of importance for symbiosis (Grote et al., 2014; Krysciak et al., 2014). Furthermore, it is also possible that the expression of rhizobial QS systems could be influenced by the host plants as these have the potential to detect AHLs as well as to generate mimics of such signaling molecules (Hirsch et al., 2003; Sanchez-Contreras et al., 2007).

The limited availability of nutrients and energy resources in such challenging environmental niches as the rhizosphere forces the competition in mixed populations (Soto et al., 2006). Since bacteria evolved the ability to communicate via QS systems, it is reasonable to speculate that these microbes evolved defense strategies to rival with each other and to disarm competitors by efficiently interfering with the key components of their QS systems (Dong et al., 2007; Gonzalez and Keshavan, 2006). Targets for such QQ processes can be either the autoinducer (AHL) synthase, the AHL signal molecule itself that can be degraded, or the cognate regulator that can be blocked by plant and/or bacterial mimics (Chajkowski and Jafra, 2009; Dessaux et al., 2011; LaSarre and Federle, 2013). Especially, the QQ strategy of AHL degradation by means of lactonases, amidases, or oxidoreductases has been found to play a significant role in obtaining a competitive advantage over other present microbes. Using this QQ strategy, plant-associated strains comprising AHLases or amidases are able to suppress other plant pathogens in their virulence activity, thereby protecting their host plant from colonization by these pathogens (Molina et al., 2003). Most surprisingly, studies carried out with transgenic plants expressing AHL-degrading proteins have been found to be resistant to QS-regulated infection by *E. carotovora* (Dong et al., 2000).

In addition to QS-mediated signaling that is important for the successful establishment of symbiosis, QQ processes may also play a key role in these rhizobia–legume associations. Therefore, the diversity of QS systems and also their availability and utilization in rhizobia might yet be another key to broad host range. Although no study has yet been published clearly demonstrating a link between host range and QQ, the ecological role of QQ proteins was already demonstrated in rhizosphere colonization studies (Krysciak et al., 2011).

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Chapter 33

Motility and Chemotaxis in the Rhizobia

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33.1 INTRODUCTION

It is generally believed that motility and chemotaxis are important in rhizobial ecology. This is based on the assumption that rhizobial cells would need to respond to specific compounds produced by the host plant, and be able to move toward the plant root system. In addition, there is a steep concentration gradient of nutrients in the soil, the highest abundance being in the rhizosphere; thus, the ability to move toward the rhizosphere would presumably be beneficial. Most of the genes involved in motility and chemotaxis in rhizobia have been studied in *Sinorhizobium meliloti*, and, within the last few years, additional information has been available in *Rhizobium etli*, *Rhizobium leguminosarum*, *Agrobacterium tumefaciens*, and *Bradyrhizobium japonicum*. With the increasing number of genome sequences available, it has also been possible to perform bioinformatic analysis to investigate the presence of motility-related genes in other rhizobial species.

33.2 THE ENTERIC MODEL SYSTEM OF CHEMOTAXIS AND MOTILITY

Most motile bacteria move through the use of flagella. Chemotaxis and flagellar motility have been studied in great detail in enteric bacteria, particularly *Escherichia coli* and *Salmonella* (recent reviews by Terashima et al., 2008; Wadhams and Armitage, 2004; Berg, 2003; Harshey, 2003; and Macnab, 2003). Thus, the enteric motility system will be used to describe, in brief, the flagellar structure and its movement. The flagellum consists of three substructures, namely: (i) the basal body that functions as a rotary motor; (ii) the filament serving as a helical propeller; and (iii) the hook that serves as a universal joint for the basal body and the filament (Terashima et al., 2008; Minamino and Namba, 2004; Berg, 2003). The basal body consists of a rod, a series of rings, the Mot proteins (stator), the switch complex, and a flagellum-specific export apparatus. The flagellar filament, on the other hand, is made up of flagellin subunits, and it extends many cell lengths from the cell surface (Minamino and Namba, 2004). Assembly of the flagellum and its movement require the synthesis of more than 50 gene products (Terashima et al., 2008).

E. coli and *Salmonella* typically exhibit five to eight peritrichous flagella randomly distributed around the cell (Harshey, 2003). Rotation of the flagella drives two types of movement: swimming and swarming motility. Swimming motility refers to the individual movement of bacteria within a liquid or semisolid environment while swarming motility corresponds to the coordinated movement of flagellated cells on top of a solid surface. Swimming motility is described in the next paragraph while rhizobial swarming will be discussed further in a different section.

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In the enteric bacteria, counterclockwise rotation of the flagella forces the peritrichous flagella to coalesce into a bundle. The coordinated movement of the flagellar bundle propels the cell, resulting in "running" or smooth swimming. The clockwise rotation of some flagella or an individual flagellum disrupts the bundle, resulting in a "tumbling" motion, consequently causing the reorientation of the cell (Harshey, 2003). In the presence of an attractant gradient, the cell runs longer up the gradient. On the other hand, the presence of a repellent or decreasing concentrations of an attractant results in more tumbles.

Environmental stimuli are sensed by several cooperating chemotaxis mechanisms in the bacteria, including phosphotransferase chemotaxis (Neumann et al., 2012), energy taxis (Alexander, 2010; Schweinitzer and Josenhans, 2010), and the best understood mechanism involving transmembrane chemoreceptors (methyl-accepting chemotaxis proteins or MCPs). *E. coli* and *Salmonella* have five MCPs, four of which (Tar, Tsr, Trg, and Aer) are shared. Tap and Tcp chemoreceptors are specific for *E. coli* and *Salmonella*, respectively (Bren and Eisenbach, 2000). MCPs consist of periplasmic and cytoplasmic domains, with the former functioning as a ligand-binding site and the latter as a signaling domain (Hazelbauer, 1988). The cytoplasmic domain is flanked by methylation regions, and it also contains a binding site for the histidine autokinase CheA. Binding of CheA to the MCP is mediated by an adaptor protein, CheW. The binding of a repellent to the periplasmic domain of the MCP results in a conformational change that signals the phosphorylation of CheA. Phosphorylated CheA transfers the phosphate group to CheY, which becomes activated. CheY-P can then interact with the switch protein (FliM), causing a reversal in the direction of the flagellar motor rotation to a clockwise mode. The clockwise rotation disrupts the flagellar bundle resulting in a tumble and a change in the direction of movement. CheY-P spontaneously dephosphorylates but its inactivation is accelerated by the dephosphatase activity of CheZ. Inactivation of CheY results in the termination of the clockwise flagellar rotation. The other important Che proteins are CheR and CheB, which methylates and demethylates the MCPs, respectively. The methylation state of the MCPs influences their efficiency to stimulate CheA autophosphorylation. Resetting the receptors then allows bacteria to sense changes within a gradient (Belas et al., 2008; Wadhams and Armitage, 2004; Bourret and Stock, 2002; Bren and Eisenbach, 2000).

A number of the enteric motility and chemotaxis elements described above are conserved in rhizobia. However, there are also major differences, including flagellar filament structure, flagellar gene organization, number of flagellin genes, and chemotactic signaling. Some of these features have been described in reviews by Poole et al., 2010 and Yost and Hynes, 2000. In this chapter, recent studies on rhizobial chemotaxis and motility (e.g., swarming motility and gene regulation) are included. In addition, results from the analysis of additional rhizobial genome sequences are presented.

33.3 FLAGELLATION AND FLAGELLAR ROTATION IN RHIZOBIA

In Bergey's *Manual of Systematic Bacteriology* (Jordan, 1984), rhizobia are generally described as motile, with peritrichous flagella, except for *B. japonicum*, which is polarly flagellated. *Mesorhizobium* is motile by one or two subpolar flagella or it could also be peritrichously flagellated (Chen et al., 2005). *R. leguminosarum* VF39SM (Tambalo et al., 2010a) and *S. meliloti* (Götz et al., 1982) can have as many as 7 and 10 peritrichous flagella (Fig. 33.1a and c), respectively; and *R. leguminosarum* 3841 has 1–3 subpolar flagella (Fig. 33.1b). *A. tumefaciens*, on the other hand, has multiple flagella that are arranged circumthecally near one end of the cell (Chesnokova et al., 1997). The motility system of *B. japonicum* is quite different from other rhizobial species, having two flagellar systems consisting of a thick subpolar flagellum and a few thin peritrichous flagella at subpolar positions (Kanbe et al. 2007). The thick flagellum is semicoiled while the thin flagella appear in a tight-curly morphology (Kanbe et al., 2007). The presence of a dual flagellar system has been described previously in *Vibrio parahaemolyticus* (McCarter, 2004; Atsumi et al., 1992). However, the *B. japonicum* flagellar system differs from that of *V. parahaemolyticus* such that both thick and thin flagella are subpolar and unsheathed, as opposed to the polar positions of the sheathed flagella in *V. parahaemolyticus* (Kanbe et al., 2007; McCarter, 2004; Atsumi et al., 1992).

Rhizobial flagella from different species differ in terms of physical properties and appearance under an electron microscope. The plain filaments of *R. leguminosarum* (Tambalo et al., 2010a; Del Bel, 2004) appear smooth (Fig. 33.1d and e). Both thick and thin lateral flagella from *B. japonicum* also resemble plain flagellar filaments (Kanbe et al., 2007). In contrast, the complex filaments of *S. meliloti* (Fig. 33.1f) and *Agrobacterium* sp. strain H13-3 (previously called *Rhizobium lupini* H13-3) have a distinct ridging pattern (Trachtenberg and DeRosier, 1987; Trachtenberg et al., 1987; Krupski et al., 1985). The complex filaments synthesized by *S. meliloti* and *Agrobacterium* sp. strain H13-3 are also rigid (Scharf et al., 2001; Sourjik and Schmitt, 1998; Sourjik and Schmitt, 1996), a property that is thought to be favorable for motility in viscous environments such as in the soil biotope (Trachtenberg and Hammel, 1992). Both the plain flagella in *R. leguminosarum* and the thin filaments of *B. japonicum* rotate unidirectionally, which is similar to the observation for complex filaments (Trachtenberg and Hammel, 1992; Götz and Schmitt, 1987). The direction of cell movement is changed by modulating the speed of

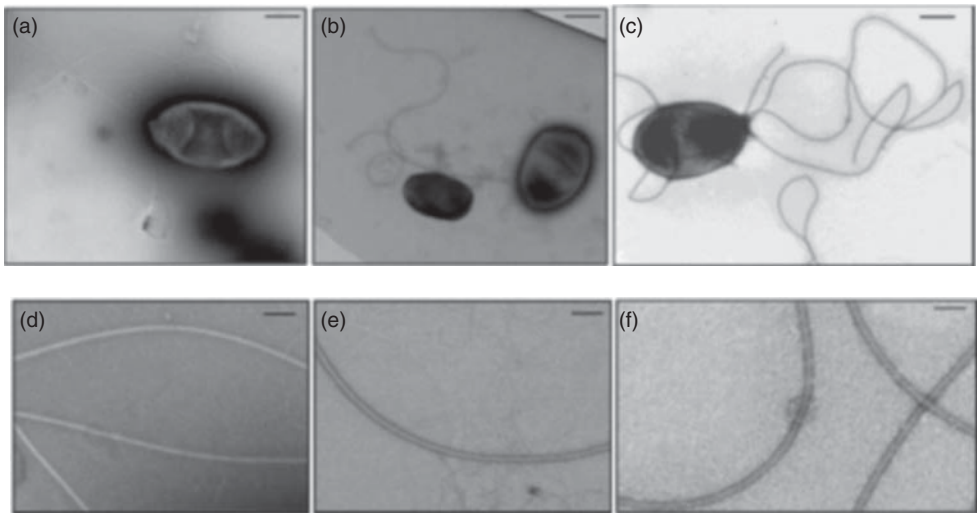

Figure 33.1 Electron micrographs of *R. leguminosarum* and *S. meliloti* 1021 flagellar filaments stained with 1% uranyl acetate. (a) VF39SM is peritrichously flagellated; (b) 3841 has a subpolar flagellum; and (c) *S. meliloti* 1021 is peritrichously flagellated. The flagellar filaments of (d) VF39SM and (e) 3841 appear to have a smooth surface and lack the ridging pattern observed on the surface of the complex flagella formed by (f) *S. meliloti* 1021. Bars: 500 nm for (a)–(c); 100 nm for (d)–(f). (Source: Tambalo et al., 2010a.)

flagellar rotation (Miller et al., 2007; Sourjik and Schmitt, 1996) so the organism turns rather than tumbles.

33.4 FLAGELLAR GENES AND THEIR GENETIC ORGANIZATION IN RHIZOBIA

The majority of the flagellar, motility, chemotaxis, and regulatory genes of *S. meliloti* (Sourjik et al., 1998), *A. tumefaciens* (Deakin et al., 1999), *M. loti* (Kaneko et al., 2000), and *R. leguminosarum* (Tambalo et al., 2010b) are located in a single region in their chromosomes. This type of genetic organization may facilitate coordinated expression of motility-related genes in the rhizobia. Analysis of the complete DNA sequences of the flagellar regulons revealed that the genes encoding nearly all of the proteins for the various rings, rod, hook, motor, and filament are present. In addition to the genes coding for the stator proteins (MotA and MotB), *motC*, *motD*, and *motE* were found in the genomes of these rhizobial species. MotC may stabilize the periplasmic domain of MotB; MotD appears to control the speed of flagellar rotation (Schmitt, 2002; Platzer et al., 1997); and MotE functions as a periplasmic chaperone for the proper folding and stability of MotC (Eggenhofer et al., 2006). The presence of the additional Mot proteins may be important in rhizobia since the flagellum functions by modulating its rotational speed rather than reversing the direction of its rotation. There are also two complete Che operons in *R. leguminosarum* and *R. etli* genomes (Miller et al., 2007). In *R. leguminosarum*, the first Che operon is located within the flagellar gene cluster, while the Che2 operon, although also found in the chromosome, is outside of the flagellar regulon. Both Che1 and Che2 operons encode the conserved set of CheAWYRB homologs that are also found in *E. coli* (Aizawa et al., 2000). *S. meliloti* also harbors two Che operons but only one has a complete set of CheAWYRB; the incomplete set of *che* genes is located on the megaplasmid pSymA and is next to genes putatively coding for pili. In the *R. leguminosarum* and *S. meliloti* genomes, there is no homolog to *cheZ*, which is consistent with previous observations that *cheZ* is found exclusively in the Betaproteobacteria and Gammaproteobacteria (Szurmant and Ordal, 2004). However, *S. meliloti* (Sourjik and Schmitt, 1998) and *R. leguminosarum* possess two or more genes coding for putative CheY proteins (Miller et al., 2007). The functions of the CheY proteins will be described later. In contrast to the rhizobial regulon described here, the *B. japonicum* genome has two sets of flagellar gene clusters and several small scattered regions with one or a few flagellar genes (Kanbe et al., 2007). The two large gene clusters contain duplicate flagellar genes, including flagellin genes that encode components of its thick and thin flagella.

33.5 CHARACTERIZATION OF RHIZOBIAL FLAGELLINS

Analysis of completely sequenced rhizobial genomes demonstrates the presence of at least two flagellin genes in each genome (Table 33.1). Among the sequenced rhizobia, *R. leguminosarum* has the largest number of predicted flagellin genes, with both biovars *viciae* (Young et al., 2006) and *trifolii* (Reeve et al., 2010) encoding seven flagellins.

Table 33.1 Number of flagellin genes and sizes of predicted proteins from sequenced rhizobia

Organisms	Number of Flagellin Genes in the Genome	Flagellin Genes	Length of Predicted Encoded Flagellins (Number of Amino Acids)
A. tumefaciens C58	4	*flaA, flaB, flaC, and flaD*	306–430
Agrobacterium sp. strain H13-3	3	*flaA, flaB, and flaD*	410–430
B. japonicum	7	*flaA, fliCI, fliCII, fliC1, fliC2, fliC3, and fliC4*	274–314 (FliA, FliCI, and FliCII) 757–763 (FliC1 to FliC4)
R. etli CFN42	6	*flaCch1, flaCch2, flaCch3, flaCch4, flaCch5, and flaCe*	301–342
R. leguminosarum bv. *viciae* strain VF39 and 3841	7	*flaA, flaB, flaC, flaD, flaE, flaF, and flaG*	301–337
R. leguminosarum bv. *trifolii* strains WSM1325 and WSM2304	7		301–332 (WSM1325) 301–389 (WSM2304)
S. meliloti 1021	4	*flaA, flaB, flaC, and flaD*	321–401
S. medicae WSM419	4	*flaA, flaB, flaC, and flaD*	321–395
*S. fredii U*SDA257	3	*flaC1, flaC2, and flaC3*	305–320
S. fredii NGR234	3	*flaA, flaB, and flaD*	305–320
M. loti MAFF303099	2	*flaA* (loci mlr2925 and mlr2927	328 and 356

In *R. leguminosarum* strains VF39SM and 3841, six flagellin genes (*flaABCDHG*) are found on the chromosome, with *flaA*, *flaB*, *flaC*, and *flaD* located within the flagellar regulon, *flaH* and *flaG* are in separate loci outside of the flagellar gene cluster; and *flaE* is plasmid-borne. In *S. meliloti*, the four flagellin genes are located in a tandem array in its 45-kb flagellar regulon (Sourjik et al., 1998). On the other hand, *flaA* and *flaB* (plus *flaC* in *A. tumefaciens*) are in tandem array but *flaD* is located on a distant portion of the flagellar regulons of *Agrobacterium* sp. strain H13-3 and *A. tumefaciens* (Scharf et al., 2001; Deakin et al., 1999).

The flagellins in *Rhizobium* (301–389 amino acids), *Sinorhizobium* (305–401 amino acids), *Agrobacterium* (306–430 amino acids), and *M. loti* (328 and 356 amino acids) are quite similar in terms of the length of the predicted protein sequences. For *B. japonicum*, FliCI (313 amino acids) and FliCII (314 amino acids) have sizes that are similar to those of the other rhizobia but the other flagellins (FliC1, FliC2, FliC3, and FliC4) are larger (757–763 amino acids). Sequence alignment of the amino acid sequences of the rhizobial flagellins revealed conserved residues at the amino- and carboxy-terminal ends. The terminal segments of flagellin proteins comprise the filament core and are essential for polymerization (Mimori-Kiyosue et al., 1997). Similar to the enteric system, the central region of the flagellins, which corresponds to the outer region of the filament, is considerably variable. In addition, the flagellin subunits cluster by genus: FlaA, FlaB, FlaC1/FlaC2, and FlaD for *S. meliloti* and *S. medicae*; FlaA/FlaCch1, FlaB/FlaCch2, FlaG/FlaC/FlaCchr3, FlaE/FlaCe, FlaH/FlaCch5 for *R. leguminosarum* and *R. etli*; FlaB and FlaC for *A. tumefaciens* C58; FlaA and FlaB for *Agrobacterium* sp. H13-3; mlr2925 and mlr2927 for *M. loti* MAFF303099; and FliC1 through FliC4 in *B. japonicum* (Fig. 33.2), suggesting that the flagellin genes did not undergo duplication or diversification until after the genera separated. Among the sequenced rhizobial species analyzed, *B. japonicum* encodes flagellins related to those of the other rhizobia only distantly.

Transcriptional studies and mutational analysis demonstrated that the rhizobial flagellar filaments are made up of more than one type of flagellin. This is contrary to the composition of the enteric flagellar filaments, which are composed of multiple copies of a single flagellin monomer, FliC (Macnab, 2003). In *S. meliloti* and *Agrobacterium* sp. strain H13-3, the flagellar filaments are assembled using four (FlaA, FlaB, FlaC, and FlaD) and three (FlaA, FlaB, and FlaD) flagellin subunits, respectively (Scharf et al., 2001). FlaA is the major flagellin subunit for both rhizobial species while FlaB, FlaC, and FlaD are secondary. Based on mutagenesis and electron microscopy imaging, FlaA and at least one of the secondary flagellins are required for the assembly of a functional flagellar filament (Scharf et al. 2001). Similarly, four flagellins (FlaA, FlaB, FlaC, and FlaD) contribute to the flagellar structure of *A. tumefaciens* (Deakin et al., 1999). Similar to *S. meliloti* and *Agrobacterium* sp. strain H13-3, mutation of *flaA* in *A. tumefaciens*

Figure 33.2 Phylogenetic tree of flagellins from *R. leguminosarum* VF39SM, *R. etli* CFN42, *S. meliloti* 1021, *S. medicae* WSM4189, *S. fredii* USDA257, *B. japonicum*, *M. loti* MAFF303099, *A. tumefaciens* C58, and *Agrobacterium* sp. H13-3.

C58 resulted in severely truncated and nonfunctional filaments. Thus, FlaA is the major flagellin, FlaB and FlaC are synthesized in lesser amounts, and FlaD is a minor component of the *A. tumefaciens* filament. In *R. leguminosarum*, three to six flagellins possibly make up its filament (Tambalo et al., 2010a). FlaA, FlaB, and FlaC are major components while FlaD and FlaG are minor components. Mutation of *flaA* in *R. leguminosarum* VF39SM results in a nonflagellated and nonmotile strain while mutation of *flaB* or *flaC* results in shorter flagellar filaments. For *B. japonicum*, four flagellins (FliC1, FliC2, FliC3, and FliC4) and two flagellins (FliCI and FliCII) possibly make

up the thick and thin flagella, respectively (Kanbe et al., 2007). Moreover, based on glycoprotein-specific staining, flagellins from *R. leguminosarum* (Tambalo et al., 2010a) and FlaA from *A. tumefaciens* (Deakin et al., 1999) are glycosylated. In the case of *R. leguminosarum*, the glycosylated flagellin(s) was(were) not identified because the subunits displayed similar migration distance on a protein gel. Sequence analysis of the seven flagellins showed the presence of glycosylation signals in each subunit. Analysis of the amino acid sequences of the other sequenced rhizobia in Table 33.1 showed putative glycosylation site(s) in at least one flagellin protein. Flagellar glycosylation has also been reported in a diverse number of bacterial species, and in some cases, it has been demonstrated to have a role in flagellar assembly (Logan, 2006). In *Azospirillum brasilense*, flagellin glycosylation is suggested to be involved in root adsorption (Moens et al., 1995), but there has been no direct evidence linking flagellin glycosylation with plant–microbe interactions. The biological role of flagellin glycosylation in the rhizosphere remains to be established.

33.6 CHEMOTAXIS

Rhizobia are chemotactic to a large number of diverse compounds as well as plant exudates (Kato and Arima, 2006; Yost and Hynes, 2000; Dharmatilake and Bauer, 1992; Barbour et al., 1991; Parke et al., 1985; Bowra and Dilworth, 1981). The exact nature of the receptors specific for these chemoattractants has not yet been determined. Nevertheless, the major elements of the chemotactic pathway in *E. coli* are present in the genomes of the rhizobial species that have been sequenced. Rhizobial species exhibit different numbers of chemotaxis operons and *mcp* genes (Table 33.2). There are two Che operons in the genome of *S. meliloti* but chemotaxis is controlled by a single operon (Schmitt, 2002). The second Che operon in *S. meliloti* is located on pSymA, and it is linked to genes involved in Type IV pilus biosynthesis, suggesting a role in twitching motility (Scharf and Schmitt, 2002). A Che operon similar to that of *S. meliloti* is conserved in *Agrobacterium* (Wright et al., 1998], *R. leguminosarum* (Che1 operon; Miller et al., 2007), corresponding to the group 1 Alphaproteobacterial Che operons described by Hauwaerts et al. (2002). *R. leguminosarum* also contains an additional complete Che operon (Che2 operon) corresponding to Che operon group 3. The *R. leguminosarum* Che1 operon is the major pathway that controls swimming bias and chemotaxis, and it is essential for competitive nodule formation. *B. japonicum* (Kaneko et al., 2002) and *R. etli* (Gonzalez et al., 2006) also have two Che operons while *M. loti* appears to have an incomplete set of *che* genes (Kaneko et al., 2000).

Bioinformatics analysis demonstrated that *S. meliloti* 1021 and *S. medicae* WSM419 have 9 *mcp* homologs each in their genomes while *M. loti* appears to have one plasmid-encoded *mcp* gene homolog. There are larger *mcp*-gene families in other rhizobia: up to 36 putative *mcp* genes in *B. japonicum* USDA110; 20 in *A. tumefaciens* C58; 27 in *R. leguminosarum*, and 28 in *R. etli* CFN42. The *mcpB* gene in *R. leguminosarum* may play a general role in chemotaxis since strains with a mutation in this receptor gene have a generalized lack of chemotactic ability. In addition, these mutants are affected in competitive nodulation. The ligand specificities encoded by the *mcp* genes in *R. leguminosarum* as well as in the other rhizobia remain to be elucidated. This could be a daunting task, since the ligands of these chemoreceptors could be unknown compounds in the rhizosphere or soil.

As mentioned earlier, there are two putative genes coding for CheY (CheY1 and CheY2) in *S. meliloti* but the CheZ protein is lacking. It is proposed that the absence of the phosphatase CheZ is compensated by CheY1 (Sourjik and Schmitt, 1998). CheY2 is the major response regulator that slows flagellar rotation upon phosphorylation by CheA (Schmitt, 2002; Sourjik and Schmitt, 1998). CheY2-P shuttles back the phosphoryl group to CheA, which then phosphorylates CheY1. Thus, CheY1 in association with CheA acts as a phosphate sink, thereby emulating the role of CheZ in the enteric bacteria (Schmitt, 2002; Sourjik and Schmitt, 1998). CheY1-P dephosphorylates spontaneously, but the rate may be too slow to provide sufficient free CheY1 (Schmitt, 2002). Very recently, Dogra et al. (2012) identified a new component (CheS) in *S. meliloti* that may play a role in accelerating CheY1-P dephosphorylation. CheS was demonstrated to bind to CheA, and phosphotransfer experiments demonstrated that CheS increases dephosphorylation

Table 33.2 Number of chemotaxis-like operons and chemoreceptors in sequenced rhizobial genomes

Organisms	Number of Chemotaxis-Like Operons	Number of Chemoreceptors (MCPs)
S. meliloti 1021	2 (1 complete set)	9
R. etli CFN42	2	28
R. leguminosarum	2	27
B. japonicum USDA110	2	36
A. tumefaciens C58	1	20
M. loti MAFF303099	1 (incomplete?)	1

of CheY1-P but not CheY2-P. Using surface plasmon resonance spectroscopy, it was further demonstrated that CheY1 binds more strongly (100-fold more) to CheA/CheS than to CheA alone. Thus, it was proposed that CheS facilitates signal termination by enhancing CheY1 and CheA interaction thereby promoting CheY1-P dephosphorylation (directly or indirectly), which results in a more efficient drainage of the phosphate sink. CheS is encoded by the major chemotaxis operon of *S. meliloti*, and CheS orthologs were also found in *S. medicae*, *A. tumefaciens*, and *R. leguminosarum* (Dogra et al., 2012).

33.7 REGULATION OF CHEMOTAXIS AND MOTILITY GENES

33.7.1 Flagellar Gene Expression in Free-Living Cells

While it is advantageous for bacteria to be able to move toward favorable environments and away from detrimental conditions, motility is energetically costly. The bacterial cell utilizes about 2% of its biosynthetic energy for the synthesis and function of the flagellum, thus flagellar gene expression is stringently regulated (Soutourina and Bertin, 2003). Similar to the enteric system, the flagellar genes of *S. meliloti* are regulated in a three-tiered hierarchy, and the genes are classified into three classes based on temporal order of expression (Fig. 33.3) (Rotter et al., 2006; Sourjik et al., 2000). The class IA genes are transcribed first and they encode VisN and VisR, which are LuxR-type proteins with characteristic DNA and ligand-binding domains. Individual mutations of *visN* and *visR* result in nonflagellated and nonmotile strains (Sourjik et al., 2000). It is believed that VisN and VisR may bind an unknown effector, and that they function as a heterodimer exerting control over the transcription of *rem* (class IB) (Rotter et al., 2006). Rem is an OmpR-like transcriptional activator that controls the expression of class II genes, which comprise basal body components (class IIA) and motility (class IIB) genes. A *rem*-mutant strain is nonflagellated and nonmotile. In *S. meliloti*, the proteins encoded by the class IIA genes *fliM* (switch protein/C-ring) and *orf38* (speculated to encode a structural component of the basal body) have been suggested to regulate the expression of the class III genes (flagellin and chemotaxis genes). Thus, similar to the enteric system, expression of the class III genes depends on the completion of the basal body structure and, possibly, flagellar export (Sourjik et al., 2000). The proteins encoded by class IIB genes (*motA*, *motB*, and *motC*) do not exert transcriptional control over the class III genes. In a different study, Hoang et al. (2008) reported that several motility genes in *S. meliloti*, including *flaF*, *flbT*, and *flaC*, are not regulated by Rem, although these genes are still controlled by VisN. In contrast to the regulation described for *S. meliloti*, the majority of the motility-related genes in *R. leguminosarum* are under VisN/R-Rem regulation (Fig. 33.3), but a subset of genes is independent of both VisN/R and Rem regulations (Tambalo et al., 2010b). The four flagellin genes (*flaA* through *flaD*), *motA*, *motB*, *che1* operon, and *mcpD*, are positively regulated by Rem through VisN/R. In strain VF39SM, the *flaE* gene that is plasmid-borne is repressed. Other chemotaxis and motility genes, which are found outside of the main motility gene cluster (*che2* operon, *flaH*, and *flaG*) or are plasmid-borne (*mcpC*), are not part of the VisN/R-Rem regulatory cascade, suggesting the presence of a secondary regulatory system in *R. leguminosarum*.

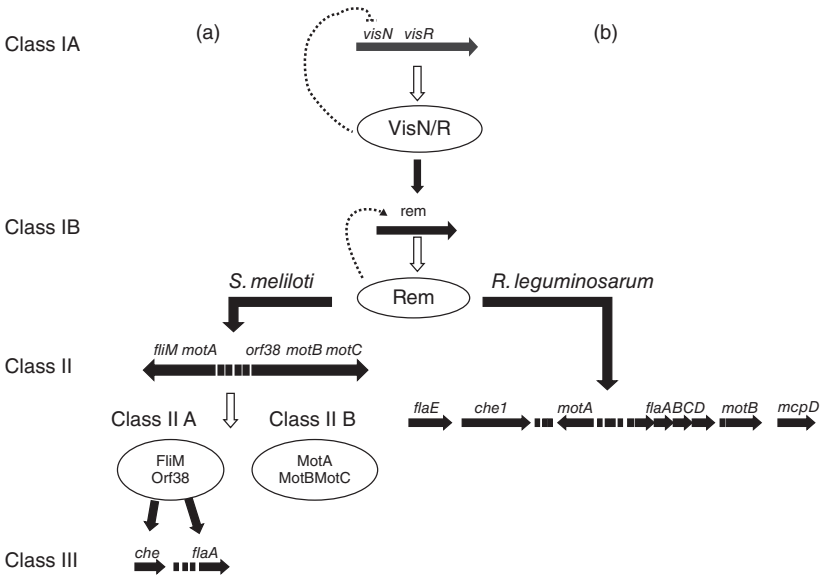

Figure 33.3 Regulation scheme of motility-related genes in (a) *S. meliloti* and (b) *R. leguminosarum*. In *S. meliloti*, the genes are classified into three classes, based on temporal level of expression. Genes are indicated as horizontal arrows and the corresponding gene products as ellipsoids. Translation to gene products is indicated by open vertical arrows. Black vertical arrows and bars indicate positive and negative regulation, respectively. Autoregulation of *visN* (in *R. leguminosarum*) and *rem* (in *S. meliloti*) is shown by dotted lines. This figure is modified from Tambalo et al. (2010b) and Rotter et al. (2006).

Real-time expression analysis showed that the expression of motility-related genes, including *flaF*, *flbT*, *cheY1*, *flaC*, *flgB*, *flgF*, and *motA*, in *S. meliloti* decreases as the cell density increases (Hoang et al., 2008). Correspondingly, electron microscopy imaging demonstrated that production of flagella is suppressed at high cell densities. Gene expression analysis further showed that the motility genes are controlled by the ExpR/Sin quorum sensing (QS) system through the VisN/VisR-Rem system. The Sin system is one of the two QS systems found in *S. meliloti* (see Chapter 37). ExpR, on the other hand, is a LuxR-type transcriptional regulator that activates exopolysaccharide (EPS) production in the presence of Sin-AHLs. Using an electrophoretic mobility shift assay, Bahlawane et al. (2008) demonstrated that AHL-bound ExpR binds to the promoter region of *visN* and inhibits its transcription. In addition, in the absence of ExpR, the expression of motility genes is lower at all growth phases. Complementation of an *expR* mutant strain with a constitutively expressed *visN* and *visR* restored the expression of motility-related genes to the wild-type levels at the early log phase of growth, suggesting that unbound ExpR and ExpR–AHL complex compete for the control of motility gene expression (Gurich and González, 2009). At low cell density, free ExpR probably activates *visN* and *visR*. As the cell population increases and the AHL concentration increases, the ExpR–AHL complex outcompetes the unbound ExpR. The ExpR–AHL complex then binds upstream of *visN* and *visR*, resulting in the repression of *visN* and *visR* expression.

Contrary to the growth-dependent expression of flagellar genes in *S. meliloti* described earlier, *R. leguminosarum* is continuously motile and flagellar genes are expressed at all growth phases (Tambalo et al., 2010b; Yost et al., 2004). This difference in temporal expression of flagellar genes in *S. meliloti* and *R. leguminosarum* suggests that although these nodule bacteria are closely related, they employ different strategies when confronted with unfavorable growth conditions such as poor growth conditions at the stationary phase. The downregulation of flagellar genes in *S. meliloti* leads to energy conservation while continued chemotaxis and motility under free-living conditions in *R. leguminosarum* may result in finding more nutrients and a more favorable environment (Tambalo et al. 2010b).

33.7.2 Flagellar Gene Expression in Bacteroids

Chemotaxis and motility are thought to be irrelevant for rhizobia once they have invaded a host plant and begun to differentiate into bacteroids. Using antiflagellin and anti-MCP antibodies, earlier studies demonstrated the absence of flagella and MCPs in bacteroids (Del Bel, 2004; Yost et al., 2004; Roest et al., 1995). Transcriptional experiments using gene fusions further demonstrated the repression of flagellin, chemotaxis, and motility genes in *R. leguminosarum* during symbiosis. Microarray experiments also showed that a flagellar gene in *M. loti* (Uchiumi et al., 2004) and 25 motility and chemotaxis genes in *S. meliloti* are downregulated in bacteroids. The signals and mechanisms for the repression of motility and chemotaxis are not established, but oxygen appears not to be the major signal, and known symbiotic regulators, such as FixL, FixK, and FnrN, may not be involved (Becker et al. 2004; Yost et al., 2004). The genes coding for the transcriptional activators VisN and Rem are also repressed during symbiosis in *R. leguminosarum*, suggesting that motility repression inside the nodules is possibly mediated by repression of VisN/R and Rem (Tambalo et al., 2010b).

33.7.3 Flagellar-mediated Swarming Motility

Swarming motility has only been described recently in the rhizobia. Swarming has been reported in *R. etli* CNPAF512 (Braeken et al., 2008; Daniels et al., 2006), *S. meliloti* (Nogales et al., 2010; Soto et al., 2002), *R. leguminosarum* bv. *viciae* (Tambalo et al., 2010c), *Agrobacterium vitis* (Sule et al., 2009), and more recently in *B. japonicum* (Covelli et al., 2013). In addition, initial characterization of *R. leguminosarum* isolates suggests that swarming could be widespread in *R. leguminosarum*; 11 out of 14 isolates tested demonstrated surface movement (Tambalo and Yost, unpublished). The specific composition of swarm media used to induce swarming differs; but they generally contain mineral salts and 0.1–1% of either of the following carbon sources: mannitol, arabinose, and glycerol. Surface movement was observed in *A. vitis* using a potato dextrose medium, which contains 1% dextrose (Sule et al., 2009). The concentration of the agar (and the type of agar in the case of *S. meliloti* QS77 (Soto et al., 2002)) appears to be critical for swarming. Surface movement was observed in agar concentrations ranging from 0.5% to 0.75%; a higher concentration (1.3%) results in swarming inhibition (Soto et al., 2002; Tambalo et al., 2010c).

33.7.4 Swarming Colony Morphology and Properties of Swarmer Cells

Rhizobial species exhibit different swarming patterns (Fig. 33.4). *R. etli* strains CFN42 (Fig. 33.4a) and CNPAF512, *S. meliloti* (Fig. 33.4b), *R. leguminosarum* 3841 (Fig. 33.4c), and *B. japonicum* (Covelli et al., 2012) swarm outwards from the point of inoculation forming short extrusions on the swarm edge. Swarming patterns in *R. leguminosarum* VF39SM (Fig. 33.4d) and *A. vitis* (Fig. 33.4e), on the other hand, appear more complex, with the formation of dendrite arms across the agar surface. In *R. leguminosarum*, the dendrite arms originate from the point of inoculation, then expand, and then branch further from the original dendrite arms. Furthermore, a cell density-dependent lag period (3–5 days) occurs before the initiation of swarming. This time frame is characterized by

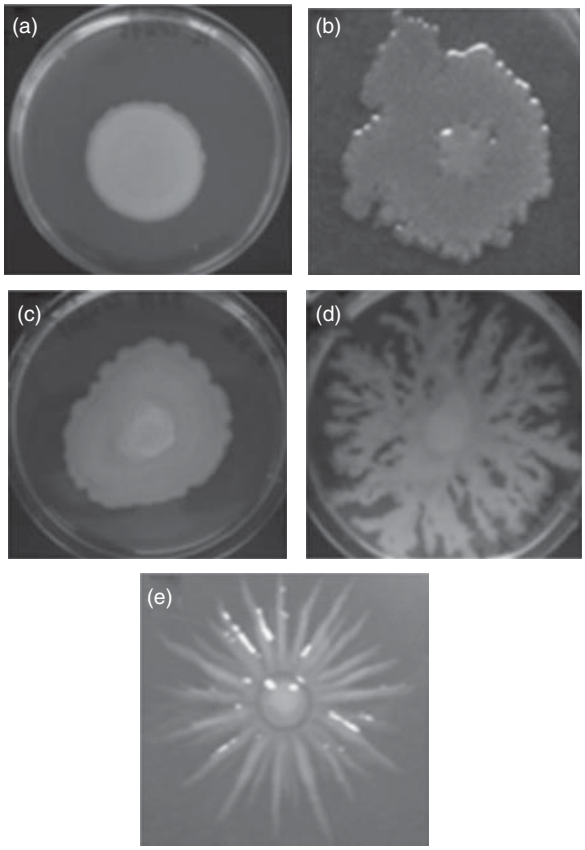

Figure 33.4 Pattern formation of rhizobial swarming colonies. (a) *R. etli* CFN42, (b) *S. meliloti* QS77 (Soto et al., 2002), (c and d) *R. leguminosarum* 3841 and VF39SM (Tambalo et al. 2010c), and (e) *A. vitis* F2/5 (Sule et al., 2009).

an increase in colony size, reflecting an increase in cell density. Thus, the lag period might be needed to reach a certain cell density to start swarming. Additionally, this phase may allow the metabolic and physiological changes associated with swarmer cells (Nogales et al., 2010; Overhage et al., 2008; Kim and Surette, 2004) and/or to build up extracellular swarm signals such as biosurfactants and extracellular slime (Verstraeten et al., 2008; Toguchi et al., 2000).

Swarming development in *S. meliloti* QS77 is accompanied by the accumulation of polysaccharides that are associated with a mucoid appearance (Soto et al., 2002). In addition, the swarming front is preceded by an extracellular slime (Tambalo et al., 2010c; Daniels et al., 2006), which may contain biosurfactants that facilitate surface movement (Verstraeten et al., 2008). Surfactants were detected in the matrix formed by *A. vitis*, but not in *R. leguminosarum*. Long-chain AHLs were also detected in the *R. etli* extracellular matrix, and these AHLs were demonstrated to have biosurfactant activity (Daniels et al., 2006). Swarming in *S. meliloti* 1021 requires the synthesis of siderophore rhizobactin 1021 (Nogales et al., 2010), which has surfactant properties and therefore probably acts as a wetting agent for surface translocation (Nogales et al., 2012). Differentiation into swarmer cells is also characterized by increased flagellation in *S. meliloti* (Soto et al., 2002), *R. leguminosarum* (Fig. 33.5a–e; Tambalo et al. 2010c), and *B. japonicum* Covelli et al., 2013). Increased flagellation in swarmer cells, relative to vegetative cells, may be necessary to overcome surface friction and/or higher viscosity created by the extracellular slime surrounding a swarming colony (Harshey, 2003). In contrast, *A. vitis* swarmer cells demonstrate similar flagellation pattern as vegetative cells, indicating that increased flagellation is not a requisite for *A. vitis* swarming (Sule et al., 2009). For *B. japonicum*, both subpolar and lateral flagella are synthesized by swarmer cells, with the latter type playing a major role in swarming. Swarmer cells were also observed to be twice as long as vegetative cells in *S. meliloti* (Soto et al., 2002) and *B. japonicum* (Covelli et al., 2013). It is believed that cell elongation results from the suppression of cell division in swarmer cells; but its role in swarming is not understood (Kearns, 2010). Another notable feature of *R. leguminosarum* swarmer cells is the formation of rafts, wherein adjacent cells are arranged parallel to their long axis (Fig. 33.5f). This rafting arrangement may promote rapid and coordinated movement of swarm cells on the agar surface (Daniels et al., 2004; Julkowska et al., 2004). *R. leguminosarum* swarmer cells were also found to be resistant to a number of different classes of antibiotics but the ecological role of antibiotic resistance in rhizobial swarmer cells remains to be elucidated.

33.7.5 Regulation of Swarming Motility

To date, little is known about the genetic control of rhizobial swarming motility. In *R. etli* CFNPAF512, swarming is regulated by the CinIR QS, which is activated in swarmer cells by its cognate AHL and other long-chain AHLs (Daniels et al., 2004). As mentioned earlier, apart from signaling function, the long-chain AHLs also act as biosurfactants. The major flagellin gene (*flaA*) and the genes coding for the transcriptional activators VisN and Rem are upregulated in *R. leguminosarum* swarmer cells, which is in agreement with increased flagellation. In *R. leguminosarum*, chemotaxis appears to be dispensable for swarming motility, which has also been reported in *Salmonella* (Mariconda et al., 2006). Mutation of the major chemotaxis operon (*che1*) and the secondary operon in *R. leguminosarum* did not significantly affect swarming motility (Tambalo, 2010). Apart from motility and QS-related genes, genes involved in polysaccharide synthesis or export and amino acid or polyamine metabolism may contribute to swarming. Individual mutations in these genes result in reduced or totally deficient swarming motility in *R. etli* CNPAF512 (Braeken et al., 2008). Furthermore, a recent transcriptomic profiling study in *S. meliloti* demonstrated that swarmer cells have different physiology compared with cells growing in liquid cultures (Nogales et al., 2010). Despite these studies,

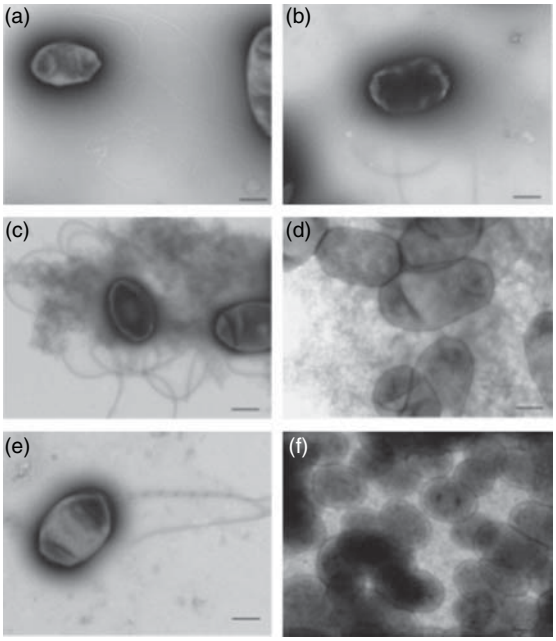

Figure 33.5 Transmission electron micrograph of *R. leguminosarum* VF39SM and 3841 vegetative and swarming cells. (a) VF39SM vegetative cells from a solid agar plate; (b) VF39SM vegetative cells taken from the center of a swarm plate; (c) VF39SM swarm cells taken from the edge of the swarm front; (d) 3841 vegetative cells from a solid agar plate; (e) 3841 swarm cells taken from the edge of the swarm front; (f) VF39SM cells taken from the edge of the swarm front. Adjacent cells are connected side by side forming rafts. Scale bar = 500 nm. (*Source*: Tambalo et al. 2010c.)

the signals and regulatory networks involved in rhizobial swarming remain poorly understood.

33.7.6 Flagellar-Independent Surface Motility in *S. meliloti*

Spreading or sliding motility has recently been reported in *S. meliloti* (Nogales et al., 2012). Sliding is a type of surface translocation that is brought by expansive forces in a growing culture in combination with special cell surface properties and surfactants that reduce friction between the cells and the substrate (Jarrell and McBride, 2008). Nogales et al. (2012) demonstrated that this type of translocation is flagellar independent; mutations in different *S. meliloti* strains that resulted in nonflagellated phenotypes did not affect surface spreading. In addition, sliding motility absolutely depends on the production of galactoglucan (EPS II) while overproduction of succinoglycan (EPS I) facilitates this movement (see also Chapter 36). It has been suggested that EPS II may serve as a hydrated milieu, providing sufficient moisture to facilitate spreading, or as a lubricant to reduce friction between cells and a semisolid surface. Twitching motility, which depends on a type IV pilus, has not been demonstrated in any rhizobia.

33.7.7 Role of Chemotaxis and Motility in Rhizobial Ecology

A number of studies have demonstrated an important role for motility and chemotaxis in the colonization of plant hosts by various rhizobial species. Although nonmotile or nonchemotactic rhizobial strains are still able to nodulate their host plants, they are usually less competitive than the wild-type strains (Yost et al., 1998; Malek, 1992; Bauer and Caetano-Anollés, 1990; Caetano-Anollés et al., 1988; Mellor et al., 1987; Soby and Bergman, 1983; Ames and Bergman, 1981). Individual mutations in the major chemotaxis gene cluster and a *mcp* gene in *R. leguminosarum* also resulted in altered competitiveness (Miller et al., 2007; Yost et al., 1998).

The role of swarming in rhizobial ecology is not yet established but this bacterial behavior may provide an ecological advantage in the rhizosphere, including colonization of plant hosts and protection from antimicrobials. During alfalfa rhizosphere colonization, *Pseudomonas fluorescens* F113 produces hyperflagellated phenotypic variants with enhanced swarming abilities, and these variants preferentially colonize distal parts of the roots (Sánchez-Contreras et al., 2002). Barak et al. (2009) also correlated swarming and alfalfa seedling colonization by identifying genes in *Salmonella enterica* that are required for swarming and colonization. In addition, alfalfa root exudates perpetuate *S. enterica* swarming. In *R. leguminosarum*, swarming motility can be enhanced or inhibited by seed exudates from Faba bean (*Vicia faba*) and lentil (*Lens culinaris*), respectively (Tambalo et al., 2014), which could affect bacterial colonization and plant host infection.

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Chapter 34

The PTS^{Ntr} System Globally Regulates ATP-Dependent Transporters in *Rhizobium leguminosarum*

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34.1 INTRODUCTION

34.1.1 Phosphotransferase Systems

Phosphotransferase systems (PTS) are widespread in the bacterial kingdom and a first description of the phosphorylation of a PTS enzyme in *E. coli* was published by Kundig et al. (1964). The best characterized systems are the so-called sugar PTS, which are facilitating the uptake and phosphorylation of incoming sugars in a wide range of Gram-negative and Gram-positive bacteria (for an extended review, see Deutscher et al., 2006). The sugar PTS is also the key player in the regulation of uptake and metabolism of different carbon sources, better known as carbon catabolite repression (CCR) in both model organisms *Escherichia coli* and *Bacillus subtilis* (Görke and Stülke, 2008). A paralogous system to the sugar PTS was first described in *E. coli* and named PTS^{Ntr} after its organization within the *rpoN* operon and a link to nitrogen metabolism (Powell et al., 1995). RpoN is the sigma factor governing the general expression of genes regulated by nitrogen availability in bacteria (see Chapters 10, 11). Both PTS consist of three core proteins that are subsequently phosphorylated in the presence of phosphoenol pyruvate (PEP). The first enzyme is called enzyme I (EI or EI^{Ntr} for PTS^{Ntr}) and rapidly autophosphorylates on a conserved histidine residue in the presence of PEP. This phosphate group is then transferred to another conserved histidine of a phosphocarrier protein (HPr or NPr for PTS^{Ntr}), which further donates the phosphate to a protein named enzyme II (EIIA or EIIA^{Ntr} for PTS^{Ntr}). In case of the sugar PTS, several EIIA proteins can be phosphorylated again on a conserved histidine. These EIIA proteins belong to EII membrane complexes of variable organization that share the ability to transport and subsequently

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phosphorylate-specific sugars (Deutscher et al., 2006). The important difference of the PTS^{Ntr} is that EIIA^{Ntr} does not interact with any transport complexes of the PTS transporter family. PTS^{Ntr} is therefore thought to have only regulatory function (Pflüger-Grau and Görke, 2010). While sugar PTSs seem to have mainly evolved in Enterobacteriales, Vibrionales, and Firmicutes, the occurrence of PTS^{Ntr} in the absence of any sugar PTS might be a regular feature of Alphaproteobacteria and Betaproteobacteria, Chlamydiae, Planctomycetes, and Xanthomonadales (Cases et al., 2007).

34.1.2 Phenotypes of PTS^{Ntr} Mutants

Mutations of Pts^{Ntr} genes cause pleiotropic phenotypes in a wide range of bacteria (summarized by Pflüger-Grau and Görke, 2010). These phenotypes are described as nitrogen-related phenotypes (metabolism, fixation, and transport) observed in *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Azotobacter vinelandii, Rhizobium etli*, and *R. leguminosarum* (Begley and Jacobson, 1994; Jin et al., 1994; Merrick and Coppard, 1989; Segura and Espin, 1998; Michiels et al., 1998; Prell et al., 2012), polyhydroxy alcanoate accumulation in *P. putida* and *A. vinelandii* (Noguez et al., 2008; Velazquez et al., 2007), carbon catabolite repression phenotypes in *P. putida* and *Sinorhizobium meliloti* (Cases et al., 1999; Pinedo et al., 2008), host interaction in *P. fluorescence* and *Legionella pneumophila* (Edelstein et al., 1999; Mavrodi et al., 2006), and regulation of K^+ transporters in *E. coli* and *R. leguminosarum* (Lee et al., 2007; Lüttmann et al., 2009; Prell et al., 2012). In many of these studies, EIIA^{Ntr} in its phosphorylated or nonphosphorylated form is thought to facilitate the regulatory effect in a direct or indirect way. Direct mechanistic evidence is only available for the regulation of K^+ transport in *E. coli* and *R. leguminosarum* (Lee et al., 2007; Lüttmann et al., 2009; Prell et al., 2012) and interaction with PhoR in *E. coli* (Lüttmann et al., 2012). Very recently, it has been shown that EIIAs phosphorylated via the PTS^{Ntr} might directly regulate the activity of central carbon metabolic enzymes such as pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Dozot et al., 2010; Pflüger-Grau et al., 2011).

However, while the sugar PTS is directly linked to sugar transport, the PTS^{Ntr} has only recently been linked to transport activities. The first report came from *Bradyrhizobium japonicum* where a PtsP (EI^{Ntr}) mutant was found to be unable to take up δ-aminolevulinic acid (ALA; King and O'Brian, 2001). ALA transport, which is facilitated via the oligopeptide transport system Opp, is abolished in a PtsP or LysC mutant of *B. japonicum* I110. LysC, which codes for an aspartokinase, is located upstream of PtsP in *B. japonicum.* PtsP and LysC interact with each other and ATP-dependent PtsP phosphorylation is reduced in the presence of LysC *in vitro* (King and O'Brian, 2001). The second example of a link of PTS^{Ntr} to transport is the direct regulation of K^+ transport activities by nonphosphorylated EIIA^{Ntr} in *E. coli* (Lee et al., 2007; Lüttmann et al., 2009) and in *R. leguminosarum* (Prell et al., 2012). In high K^+ medium, an *E. coli ptsN* (EIIA^{Ntr}) mutant has difficulties to grow because nonphosphorylated EIIA^{Ntr} inhibits the low-affinity K^+ transporter Trk via protein–protein interaction with TrkA (Lee et al., 2007), which leads to an accumulation of high intracellular K^+ levels. In contrast, in low K^+ medium, nonphosphorylated EIIA^{Ntr} stimulates the phosphorylation of the two-component system KdpDE, which leads to an increased transcription of the high-affinity K^+ transporter KdpFABC in *E. coli* (Lüttmann et al., 2009).

Here, we describe a link of the PTS^{Ntr} of *R. leguminosarum* to the ATP-dependent ABC transporter superfamily and the P-type ATPase K^+ transporter KdpABC.

34.2 RESULTS AND DISCUSSION

34.2.1 EI^{Ntr} Is Required for Full Activation of ABC Transport in *R. leguminosarum* 3841

While screening for Tn5 mutants of *R. leguminosarum* 3841 (Rlv3841) with altered surface phenotypes, a dry colony morphology on TY agar plates could be linked to a *ptsP* mutation (Prell et al., 2012). The mutant strain PtsP107 formed small, flat, and dry colonies in contrast to the large and mucous colonies formed by the wild-type Rlv3841.

We started to investigate a link between PTS^{Ntr} and ABC transport because of three reasons: (i) mutation of *ptsP* or *lysC* in *B. japonicum* I110 leads to the inactivation of Opp, an oligopeptide transporter of the ABC transporter superfamily (King and O'Brian, 2001); (ii) exopolysaccharide secretion, which makes *R. leguminosarum* strains extremely mucous, is known to involve ABC transporter components (Becker et al., 1995; Paulsen et al., 1997; Vanderlinde et al., 2010); and (iii) the two general amino acid ABC transport systems Aap and Bra are posttranscriptionally regulated by an unknown mechanism and upon an unknown signal in a glutamine oxoglutarate aminotransferase (GOGAT) mutant background in Rlv3841 (Mulley et al., 2011).

When the *ptsP* mutant PtsP107 was grown in AMS minimal medium with 10 mM glucose and NH_4Cl and transport rates of five different solutes, which are known to be exclusively taken up via at least seven ABC transport systems, were compared to transport levels of wild-type Rlv3841, a reduction of transport rates between 50% and 80% was observed (Fig. 34.1; Prell et al., 2012). In contrast, the uptake of succinate, which is facilitated via the proton-coupled DctA transport system, was unaffected. Consistent with this, PtsP107 grew very poorly on AMS glucose/glutamate plates because glutamate is taken up

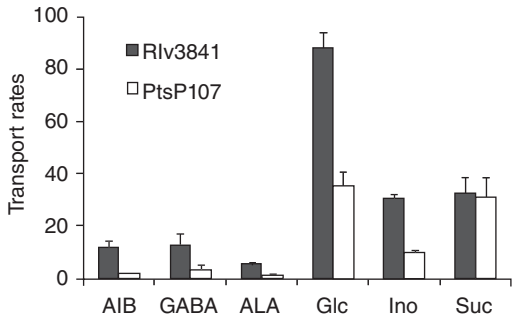

Figure 34.1 Transport rates of wild type Rlv3841 and its *ptsP*::Tn*5* mutant PtsP107. α-aminoisobutyric acid (AIB), γ-aminobytyric acid (GABA), δ-aminolevulinic acid (ALA), glucose (Glc), and inositol (Ino) are taken up via published ABC systems; succinate (Suc) is taken up via the proton coupled DctA transporter (for details see Prell et al., 2012). Rates are given in nanomole solute per min milligram per protein. (*Source*: Modified from Prell et al., 2012.)

via the ABC transport systems Aap and Bra in Rlv3841. These results suggested that PtsP is required for general ABC transport activation and posed the question whether the entire PTS^{Ntr} phosphorylation cascade is linked to that phenomenon.

34.2.2 Identification of Other PTS^{Ntr} Genes in the Rlv3841 Genome

The Rlv3841 genome contains at least 19 genes that encode proteins with homology to PTS components. One protein, LysC, should be added to this list, because it interacts with PtsP in *B. japonicum* I110 (King and O'Brian, 2001).

34.2.2.1 LysC. Upstream of PtsP (EI^{Ntr}, RL4283) orientated in the same direction, a *lysC* gene, was found (RL4284) in the Rlv3841 genome that very likely encodes an aspartokinase as demonstrated in *B. japonicum* (King and O'Brian, 2001). Mutation of the Rlv3841 *lysC* gene did not result in a surface phenotype identical to the *ptsP* mutation. When tested for amino acid transport, only a minor but significant reduction of transport rates could be measured in the *lysC* mutant RU4192, and bacterial two-hybrid experiments could not verify a protein–protein interaction between LysC and PtsP as shown in *B. japonicum* (Prell et al., 2012). In *B. japonicum* I110, a *lysC* or *ptsP* mutation has the same effect on Opp transport. Therefore, we concluded that there is no strong link between LysC and PtsP in *R. leguminosarum* in contrast to *B. japonicum*. Mutation of *lysC* in Rlv3841 also did not cause auxotrophy for the strain, which poses the question how *R. leguminosarum* generates lysine, threonine, and methionine, which require aspartyl β-phosphate as a precursor. Investigation of the Rlv3841 genome did not produce an obvious *lysC* homolog. However, the location of *lysC* upstream of *ptsP*, sometimes separated by one or few genes, seems to be a general feature of Alphaproteobacterial genomes with only very few exceptions (analysis of 197 genomes of the MicrobeOnline database; unpublished observation).

34.2.2.2 EIIA^{Ntr}. The next observation in Alphaproteobacterial genomes is that while *ptsN* coding for EIIA^{Ntr} is located downstream of *rpoN*, a *ptsO* gene coding for NPr is missing. NPr, the intermediate phosphocarrier protein of PTS^{Ntr}, is encoded within the *rpoN* operon of many other bacterial families. Surprisingly, *ptsN* (RL0425; PtsN1) has an additional copy within the Rlv3841 genome (pRL110376; PtsN2) located downstream of the K^+ transporter genes *kdpABCDE*. Mutation of *ptsN1* (RL0425) or *ptsN2* (pRL110376) alone did not result in a surface phenotype, but the double-mutant LMB272 (*ptsN1*::ΩSpec *ptsN2*::ΩTet) showed a dry surface phenotype on TY plates, although slightly less pronounced than that of PtsP107 (Prell et al., 2012). This surface phenotype of LMB272 was accompanied by a substantial reduction (~65%) of amino acid transport rates. The *ptsN1* single-mutant LMB271 also showed transport rates significantly reduced by ~40%, suggesting that PtsN1 is contributing stronger to the described phenotypes.

34.2.2.3 NPr. An obvious candidate for the intermediate phosphocarrier protein NPr was initially missing in the Rlv3841 genome. HPr and NPr share an amino acid identity of only 30% in *E. coli* K12. However, two HPr homologs were found on the Rlv3841 chromosome: RL0032 showed 37% and 33% identity to NPr and HPr from *E. coli* K12, while RL2903 had 28% and 31% identity, respectively. RL0032 is part of an operon that is also conserved in a majority of Alphaproteobacteria as mentioned by others before (Boel et al., 2003; Hu and Saier, 2002; Pinedo and Gage, 2009). This operon consists of genes encoding a two-component regulatory system: ChvIG, an HPr kinase/phosphatase (HprK with a conserved truncation; Boel et al., 2003), a single EIIA^{Man} protein lacking any membrane domains and an HPr homolog. This operon has attracted significant attention in the past. Mutation of *chvI* or *chvG* homologs has produced pleiotropic phenotypes in *Agrobacterium tumefaciens*, *Brucella abortus*, *Sinorhizobium meliloti*, and *Rhizobium leguminosarum* (Belanger et al., 2009; Charles and Nester, 1993; Sola-Landa et al., 1998; Vanderlinde and Yost, 2012). All the mentioned species require this system to successfully invade their respective hosts, and the growth phenotypes of *Sinorhizobium meliloti* and *Rhizobium leguminosarum chvIG* mutants are especially dramatic. In both species, *chvG* mutants are not able to grow in liquid broth or on full medium plates. Growth is limited to minimal medium plates with a reduced

selection of carbon and nitrogen sources (Belanger et al., 2009; Vanderlinde and Yost, 2012), and selection of those mutants has been challenging. Mutation of HprK, EIIA^{Man} (ManX), and HPr in *S. meliloti* 1021 are all linked to succinate-mediated catabolite repression (SMCR; Pinedo et al., 2008; Pinedo and Gage, 2009).

Mutation of RL0032 in Rlv3841 resulted in an identical surface and transport phenotype as mutation of *ptsP* (Untiet et al., 2013). This is a clear indication that RL0032 is indeed NPr the phosphocarrier protein of PTS^{Ntr} in *R. leguminosarum*, and that the general activation of ABC transport involves the whole PTS^{Ntr} phosphorylation cascade.

34.2.2.4 Nontransporting Dihydroxyacetone EII Complexes.

The second above-mentioned HPr homolog RL2903 is part of one of three dihydroxyacetone (DHA) PTS gene clusters (RL1749-52; RL2900-06; pRL120386-7), which make up the majority of PTS genes in Rlv3841. DHA PTS is responsible for DHA metabolism via PTS phosphorylation but not transport (Deutscher et al., 2006). According to Barabote and Saier (2005), these clusters might form a fully functional DHA PTS, which has never been reported to interact with sugar PTS or PTS^{Ntr}. We did not test Rlv3841 for growth on DHA, but mutation of RL2903 in Rlv3841 did not result in a surface phenotype or a reduction of ABC transport activity (Untiet et al., 2013).

Because of the conserved arrangement of (i) ***ptsP*** with *lysC*; (ii) ***npr*** with *chvIG*, *hprK*, and *manX*; and (iii) ***ptsN*** with *rpoN* but without *npr* (*ptsO* in *E. coli*), these genes probably express the typical PTS^{Ntr} of Alphaproteobacteria.

34.2.3 Phosphorylation of the PTS^{Ntr} Proteins

Because of the identical phenotypes of EI^{Ntr} (PtsP) and NPr mutants and the very similar phenotype of the EIIA^{Ntr} (PtsN1/2) double mutant in Rlv3841, we concluded that the regulating protein that activates ABC transport in a direct or indirect way must be phosphorylated EIIA^{Ntr} (Prell et al., 2012). Additionally, overexpression of PtsP in wild-type Rlv3841 increased amino acid transport rates, suggesting activation. Complementation of the respective mutants with PtsP, NPr, PtsN1, or PtsN2 on plasmids always fully restored the wild-type surface and transport phenotypes (Prell et al., 2012; Untiet et al., 2013). Complementation with nonphosphorylatable mutant versions of PtsP (PtsPH367A) and NPr (NPrH17A) on the low-copy number plasmid pRK415 did not change the surface phenotype nor activate ABC transport above the respective mutant levels (Untiet et al., 2013). This indicates that indeed phosphorylation of the PTS^{Ntr} proteins is required for ABC transport activation. However, overexpression of a nonphosphorylatable PtsN1 (PtsN1H66A) on the mid-copy number plasmid pBBRMCS5 in LMB272 (*ptsN1*::ΩSpec *ptsN2*::ΩTet) did complement both the surface and transport phenotype similar to the wild-type version (Prell et al., 2012). We concluded that PtsN1H66A must be a weak activator of ABC transport when overexpressed, and the experiment has to be repeated with low and single copy versions of PtsN1H66A.

We also purified EI^{Ntr} (PtsP) as a 6His-MBP (maltose-binding protein)-PtsP version and NPr as 6His-NPr from *E. coli* BL21 cells. We could show that 6His-MBP-PtsP readily autophosphorylates in the presence of ^{33}P-PEP but not in the presence of ^{33}P-ATP (Untiet et al., 2013). The phosphate on 6His-MBP-PtsP was then donated to 6His-NPr. The donation further to PtsN1, PtsN2, and possibly ManX (EIIA^{Man}) has still to be demonstrated. Phosphorylation of ManX and PtsN (EIIA^{Ntr}) by NPr was recently demonstrated for the protein homologs in *Brucella melitensis* (Dozot et al., 2010) and is therefore likely to also occur in Rlv3841. Indirect evidence for a possible phosphorylation of EIIA^{Man} by NPr (called HPr in that study) comes also from the work in *S. meliloti* where both proteins are linked to SMCR phenotypes (Pinedo et al., 2008).

34.2.4 Role of the EI^{Ntr} N-terminal GAF Domain

One unique characteristic that separates EI^{Ntr} from EI is an N-terminal extension of ~180aa harboring a GAF domain. GAF domains are well known in the NifA transcriptional regulator family, which is involved in sensing of the nitrogen status in nitrogen-fixing bacteria (Little and Dixon, 2003). GAF domains are ligand-binding domains and it is tempting to speculate that EI^{Ntr} GAF domain regulates phosphorylation of the PTS^{Ntr} cascade. However, a ligand binding to the EI^{Ntr} GAF domain still awaits its discovery.

Removing the GAF domain from the Rlv3841 EI^{Ntr} (PtsP) protein still allowed activation of ABC transport (Untiet et al., 2013). Consistent with this, the truncated PtsPΔGAF also still complemented the dry surface of the *ptsP* mutant PtsP107. This suggests that a ligand binding to GAF might be a negative regulator of PTS^{Ntr} phosphorylation.

34.2.5 A Second Level of Regulation of PTS^{Ntr}

In *Bacillus subtilis*, HprK is able to phosphorylate a conserved serine residue of HPr. This serine phosphorylation reduces the EI-mediated histidine phosphorylation of HPr and is a strong regulator of CCR (Görke and Stülke, 2008). HprK is absent in *E. coli* but present as a truncated protein in Alphaproteobacteria (Boel et al., 2003). In *Brucella melitensis*, it has been shown that HprK can phosphorylate NPr on the conserved serine residue. Prevention of that serine phosphorylation increases the levels of histidine phosphorylation

of NPr and therefore $EIIA^{Ntr}$ phosphorylation (Dozot et al., 2010). In *S. meliloti*, *hprK* mutation results in strong growth deficiencies, loss of SMCR, and loss of effective nodulation of alfalfa plants (Pinedo and Gage, 2009). Almost identical phenotypes were observed when an *npr* mutant (called *hpr* in that study) was complemented with an NPr variant, lacking the conserved serine residue. This indicates that the hprK mutant phenotypes are mediated via phosphorylation of NPr.

Complementation of an *npr* mutation in Rlv3841 with an NPr variant lacking the conserved serine residue also resulted in strong growth reduction of the strain similar to *S. meliloti*. Additionally, the strain turned very mucous (unpublished data). It is tempting to speculate that the very mucous phenotype results from increased ABC transport-dependent EPS secretion mediated via increased $EIIA^{Ntr}$ phosphorylation.

Altogether the presence of HprK and its ability to regulate NPr histidine phosphorylation in Alphaproteobacteria opens a new and exiting second level of $EIIA^{Ntr}$ (PtsN) and $EIIA^{Man}$ (ManX) regulation, which might involve direct interaction with carbon metabolic enzymes.

34.2.6 Regulation of the High-Affinity KdpABC Transporter

KdpABC is a P-type ATPase that is controlled transcriptionally by the two-component regulatory system KdpDE. In *E. coli*, nonphosphorylated $EIIA^{Ntr}$ (PtsN) stimulates the phosphorylation of KdpD, which in turn activates KdpE (Lüttmann et al., 2009). Phosphorylated KdpE increases transcription of *kdpFABC* mRNA and, therefore, KdpFABC activity. However, in *E. coli*, the main activator of KdpDE phosphorylation is the K^+ level of the medium. In contrast, *kdpABC* expression is under the strict control of PtsN in Rlv3841. A *kdpA* mutant (RU4393) abolishes growth in medium with K^+ levels below 100 µM (Prell et al., 2012). The *ptsN1/2* double-mutant LMB272 shows exactly the same phenotype, while wild-type Rlv3841 and the *ptsP* mutant PtsP107 grow down to K^+ levels of 1 µM. This indicates that PtsN is strictly required for *kdpABC* expression. Q-RT-PCR data support this hypothesis, showing that *kdpAB* expression is constitutively upregulated in PtsP107 compared to wild-type Rlv3841 (Prell et al., 2012) and downregulated in LMB272 (unpublished data). Constitutive upregulation in PtsP107 indicates that nonphosphorylated PtsN is the Kdp-activating variant identical to the situation in *E. coli*. However, a homolog of the low-affinity Trk K^+ transporter, which is inhibited by nonphosphorylated PtsN in *E. coli* (Lee et al., 2007), is absent in *R. leguminosarum*. Low-affinity K^+ transport is likely facilitated via the Kup systems in Rlv3841, which have so far not been implicated in PTS^{Ntr} regulation.

34.2.7 Inactivation of ABC Transport at Low K^+ Levels

The existing evidence that EII^{Ntr} (PtsN) is regulating several members of the ATP-dependent ABC transporter superfamily, and on the other hand, the essential P-type ATPase KdpABC suggests a general regulation of ATP-dependent transport activities (Prell et al., 2012). It also allows the prediction that under strong K^+ limitation, where *kdpABC* transcription is induced by nonphosphorylated PtsN, ABC transport, that requires phosphorylated PtsN should be limited. When tested, wild-type Rlv3841 transported amino acids under strong K^+ limitation (1 µM), where PtsN must be nonphosphorylated at rates comparable to the rates of the *ptsP* mutant PtsP107, where PtsN cannot become phosphorylated (Untiet et al., 2013). Consistent with that, Rlv3841 growth in glucose/glutamate medium at 1 µM K^+ levels was significantly slower than growth in glucose/ammonium medium, while growth rates at non-limiting conditions (1 mM K^+) are identical.

This is to our knowledge the first report where PTS^{Ntr} dictates the regulation of essential cellular functions, namely K^+ homeostasis and ABC transport activities at limiting K^+ medium levels (Untiet et al., 2013).

A model of PTS^{Ntr} regulation in Rlv3841 that summarizes our findings and proposed functions published by others is shown in Figure 34.2.

34.3 CONCLUSIONS

PTS are historically some of the most intensively studied regulatory systems in bacteria. Although sugar PTS are the best investigated, PTS^{Ntr} is probably the most widespread PTS among the different bacterial families. PTS^{Ntr} was initially thought to regulate aspects of nitrogen metabolism or probably mediate between carbon and nitrogen metabolism. In *E. coli*, nitrogen metabolic phenotypes disappeared when *ptsN* mutant strains with an intact *ilvGM* operon coding for acetohydroxy acid synthase II (AHAS II, an enzyme of the branched chain amino acid biosynthetic pathway) were analyzed (Reaves and Rabinowitz, 2011). AHAS I, encoded by *ilvBN*, is sensitive to increased K^+ levels in contrast to AHAS II, and the authors claim that the nitrogen-related *ptsN* mutant phenotypes are a result of increased intracellular K^+. However, nitrogen-related phenotypes in other proteobacteria could be a result of ABC transport inactivation.

The only verified mechanistic actions of the PTS^{Ntr} are inhibition/activation of K^+ transport (Lüttmann et al., 2009; Prell et al., 2012; Lee et al., 2007) and PhoR regulation (Lüttmann et al., 2012) mediated via nonphosphorylated $EIIA^{Ntr}$. Additionally, EIIAs might regulate the activity of central carbon metabolic enzymes (Dozot et al., 2010; Pflüger-Grau et al., 2011).

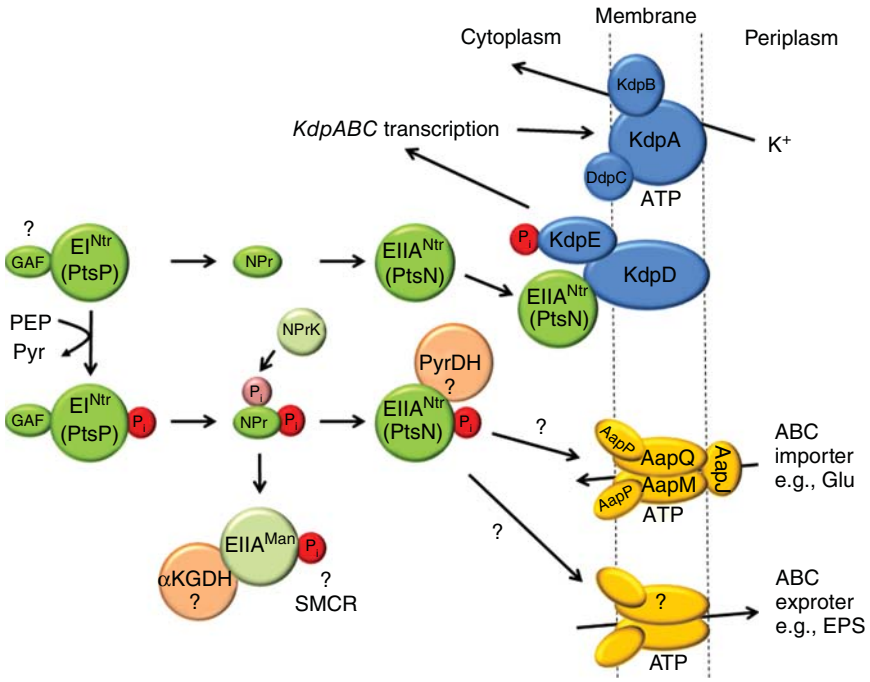

Figure 34.2 Model of PTS^{Ntr} regulation in *Rhizobium leguminosarum* with possible parallels to *Sinorhizobium meliloti*, *Brucella melitensis*, and *Pseudomonas putida*. Nonphosphorylated PTS^{Ntr} induces KdpABC by activation of KdpDE via protein–protein interaction (upper part) and phosphorylated PTS^{Ntr} activates ABC transport systems via an unknown mechanism (lower part). The regulating protein is in both cases EIIA^{Ntr}. Additionally, EIIA^{Man} regulates succinate-mediated catabolite repression (SMCR) in *S. meliloti* via an unknown mechanism. Possible cross-regulation of NPr histidine phosphorylation via regulatory serine phosphorylation by NprK (HprK) is indicated. Possible interaction with carbon metabolic enzymes such as pyruvate dehydrogenase (PyrDH in *P. putida*; Pflüger-Grau et al., 2011) and α-ketoglutarate dehydrogenase (αKGDH in *B. melitensis*; Dozot et al., 2010) are added. Question marks (?) indicate unknown ligands (EI^{Ntr} GAF), unknown mechanistic interactions (ABC transport regulation and SMCR), or not verified regulatory effects in Rlv3841 (metabolic enzymes and SMCR). (*Source*: Modified and extended from Prell et al., 2012.)

From our studies, it emerges that PTS^{Ntr} also regulates ABC transporter activities. Rhizobia are especially rich in ABC transport systems, probably as an adaptation to their oligotrophic lifestyle in soil and the rhizosphere (Mauchline et al., 2006). ABC transporter and P-type ATPases, such as KdpABC, are both primary transport systems with high affinities that use ATP hydrolysis to take up solutes against a gradient. We therefore speculate that PTS^{Ntr} might regulate ATP-dependent processes. This might also involve the balancing of carbon and nitrogen metabolism. Altogether we speculate that PTS^{Ntr} might in fact be a PTS^{ATP} or a PTS^{adenylate}.

GLOSSARY

CCR carbon catabolite repression

SMCR succinate-mediated catabolite repression

EI^{Ntr} enzyme 1 of PTS^{Ntr}, synonym PtsP, homolog of EI of sugar PTS

NPr phosphocarrier protein of PTS^{Ntr}, synonym PtsO, homolog of HPr of sugar PTS

EIIA^{Ntr} enzyme 2 of PTS^{Ntr}, synonym PtsN, homolog of EIIAs of sugar PTS

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Section 7

Nitrogen Fixing Organisms, the Plant Rhizosphere and Stress Tolerance

Chapter 35

Actinorhizal Plant Root Exudates Alter the Physiology, Surface Properties, and Plant Infectivity of *Frankia*

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35.1 INTRODUCTION

Among the soil-dwelling actinobacteria, members of the genus *Frankia* are distinguished by their ability to form symbiotic nitrogen-fixing associations with a variety of woody dicotyledonous plants (termed actinorhizal plants) representing eight different plant families of angiosperms (Benson and Silvester, 1993; Schwencke and Carú, 2001; Perrine-Walker et al., 2011; Wall, 2000). *Frankia* exists either in a free-living state in the soil or in symbiosis with actinorhizal plants (Benson and Silvester, 1993; Chaia et al., 2010; Schwencke and Carú, 2001). Actinorhizal plants are ecologically important as pioneer community plants; distributed worldwide in a broad range of ecological conditions; and have economic significance in land reclamation, reforestation, soil stabilization, landscaping, and fuel. The symbiosis allows actinorhizal plants to colonize harsh environmental terrains.

Symbiotic interactions between *Frankia* and the host plant are not well understood at a molecular level. At the morphological level, the actinorhizal symbiosis has been well studied. Root nodule formation in actinorhizal plants occurs in several stages that require a series of interactions between the bacteria and host root cells (Perrine-walker et al., 2011; Obertello et al., 2003; Wall and Berry, 2008). To initiate the symbiosis, the bacteria must recognize its host plant, attach to the surface, and penetrate the root. *Frankia* infects host plants by two distinct routes: intracellular penetration of a plant epidermal cell at a bend in a deformed root hair and intercellular penetration *of* root epidermis cells and cortex cells. Following penetration, an infection thread is established that is composed of a mass of bacteria encapsulated by polysaccharide matrix produced by the host. As the infection progresses, limited cell divisions occur close to the infection site generating a prenodule. Some prenodule cells become infected by *Frankia* and differentiate to fix nitrogen (Laplaze et al., 2000). Concomitantly, cell divisions are induced in the pericycle in front of a xylem pole. These divisions give rise to a nodule lobe primordium. As the nodule lobe primordium grows, the nodule cortex becomes infected intracellularly by *Frankia* hyphae coming from the prenodule.

Host recognition by the microbe and the identification of the beneficial microbe partner by the host plant are central to the establishment of a solid mutualism. This interaction implies cell-to-cell communication mechanisms. Information on these initial stages of interaction between the actinorhizal host plant and their symbiont, *Frankia*, in the soil is lacking. Elucidation of the *Frankia* genomes (Normand et al., 2007; Sen et al., 2013; Ghodhbane-Gtari et al., 2013) has revealed the absence of the common *nod* genes found in the *Rhizobium*–legume symbiosis. This result suggests that the actinorhizal symbiosis uses novel signal compounds during the infection process. The signaling molecules of actinorhizal plants would be perceived by *Frankia* prior to infection and would alter their physiology. (Beauchemin et al., 2012) addressed this problem by analyzing the effects of host plant root exudates on *Frankia* physiology. *Frankia* sp. strain CcI3 and *Casuarina cunninghamiana* were selected as a model system because of several reasons including that the narrow-host-range symbiont *Frankia* sp. strain CcI3 genome was sequenced (Normand et al., 2007). As a follow-up, the effects of root exudates on other *Frankia* strains were also examined.

35.2 MATERIALS AND METHODS

Frankia cultures were grown and maintained as described previously (Beauchemin et al., 2012; Tisa et al., 1999). The following *Frankia* strains were used in this study: strains CcI3, ACN14a, QA3, CN3, EUN1f, and EAN1pec.

Plant growth conditions were as described previously (Beauchemin et al., 2012). Seeds from *C. cunninghamiana*, *Elaeagnus angustifolia, Alnus glutinosa*, *Myrica cerifera*, *Hippophae rhamnoides*, and *Betula pendula* were used in this study. Briefly, seeds were sterilized by hydrogen peroxide treatment and washed with sterile deionized water. The surface sterilized seeds were germinated in perlite at 28 °C with a 16 h light period and 8 h dark period. Figure 35.1 outlines the process for the production of plant root exudates. At 7–12 days after seed germination, five plant seedlings were aseptically transplanted from the perlite to an aluminum screen in a magenta box suspended over 50 ml of 1/4 Hoagland's modified basal salt solution (1/4 HS). The plants were incubated at 28 °C with a 16 h light period. For each magenta box, spent growth medium was replaced weekly with fresh sterile 1/4 HS medium. The spent plant growth medium (root exudates) was collected monthly and filter-sterilized.

The physiological effect of root exudates on *Frankia* cultures was determined as described previously (Beauchemin et al., 2012). Briefly, cultures exposed to root exudates were assayed by multiple methods. First, hyphal curling was observed under phase contrast microscopy. Second, cell surface changes were examined by FTIR spectroscopy (Furnholm et al., 2012) and by Congo red dye binding (Beauchemin et al., 2012; Etienne et al., 2002)

35.3 RESULTS AND DISCUSSION

35.3.1 Effect of Root Exudates on *Frankia* Growth

Aqueous root exudates were used in this study and represent the natural exudates of the plant root. Although

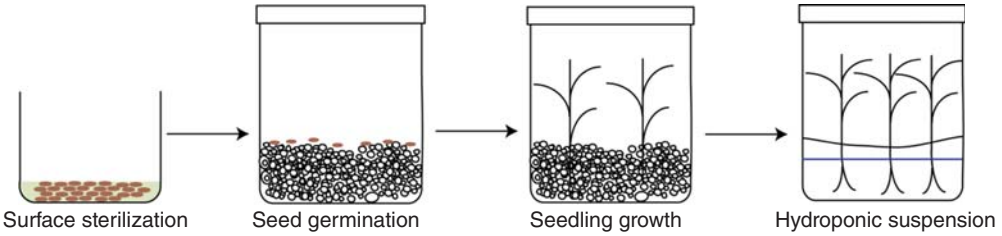

Figure 35.1 Diagram of workflow to grow aseptic seedlings for root exudates collection and nodulation studies.

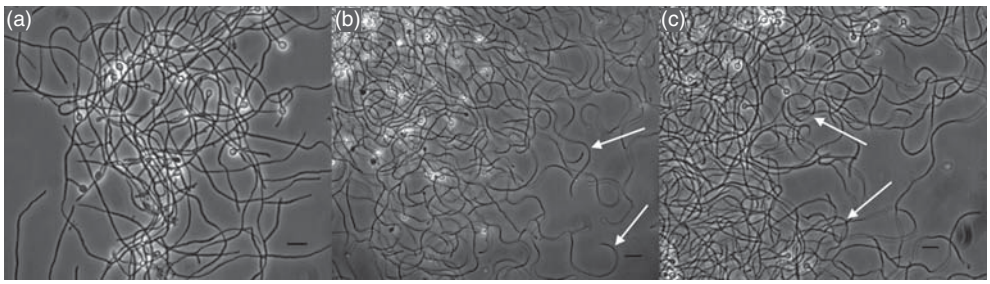

Figure 35.2 Hyphal curling response of *Frankia* CcI3 upon exposure to host root exudates. *Frankia* CcI3 cells incubated for 14 days in (a) 1/4 HS (N_2) medium (control), (b) soil-grown 2-month-old *C. cunninghamiana* root exudates, or (C) axenic 1-month-old *C. cunninghamiana* root exudates. Cultures were observed under phase contrast microscopy. Bar = 10 µm. Arrows point to regions showing curling effect. (From Beauchemin et al., 2012 with permission.)

flavonoid compounds have been identified in actinorhizal plants (Benoit and Berry, 1997; Popovici et al., 2010; see Chapter 50), we did not want to exclude any possible aqueous or organic plant signaling molecules. The exudates were collected over time from our magenta box system (Fig. 35.1). Root exudates from plants grown under both nitrogen-sufficient and nitrogen-deficient conditions were collected.

Root exudates alone were unable to support growth of *Frankia* sp. strain CcI3. In growth medium with root exudates in the absence of additional carbon sources, no growth yields were observed. In the presence of a carbon source, growth yield increased in response to root exudates and was more affected by dry weight changes than protein content. These results suggest that root exudates influence *Frankia* physiology by affecting other cellular components.

35.3.2 Root Exudates Caused Changes in *Frankia* Surface Properties and Hyphal Curling

The morphological effects of root exudates on *Frankia* physiology were examined. *Frankia* cultures exposed to root exudates showed a hyphal curling response (Fig. 35.2). The tips of the hyphae were curled or bent in response to the root exudates. Those cultures exposed to Hoagland's medium (control) did not exhibit this response.

The curling response was observed with root exudates from plants grown under both nitrogen-sufficient and nitrogen-deficient conditions. The age of the plants that root exudates were collected did not affect the curling response. The curling response was specific to the host plant root exudates, and *Frankia* sp. strain CcI3 cultures exposure to nonhost plant root exudates failed to respond.

Other surface properties were investigated. Congo red dye absorption was reduced for cultures exposed to host plant root exudates. Congo red dye binding has been aligned with changes in lipids and lipoproteins for mycobacteria (Cangelosi et al., 1999). These changes were confirmed by FTIR analysis. Exposure of *Frankia* cultures to plant host root exudates caused several changes in the spectral patterns that are indicative of alterations in fatty acids, fatty acids and proteins, and cell wall carbohydrates. In addition, the cells exposed to root exudates were more difficult to pellet than the control cells. Altogether, this observation and the Congo red and FTIR results indicate that the surface property changes occurred in response to the plant host nutrients and/or signaling molecules.

35.3.3 Root Exudates Exposure Caused Changes in Plant Infectivity

The effects of host plant root exudates on the *Frankia* surface property changes suggest major modifications in the bacterial exterior that could influence plant–microbe interaction.

Frankia cultures were pre-exposed to host plant exudates and tested for plant infectivity. Figure 35.3 shows the results of those experiments. Cultures pre-exposed to the root exudates produced root nodules 15 days after inoculation, while those untreated control cells initiated nodulation after 19.6 days.

35.3.4 Effects of Actinorhizal Root Exudates on Other *Frankia* Strains

Frankia sp. strain CcI3 is a narrow-host-range symbiont. We were interested in extending our study to other members of the *Frankia* including broad-host-range symbionts. The effects of several different root exudates were tested on

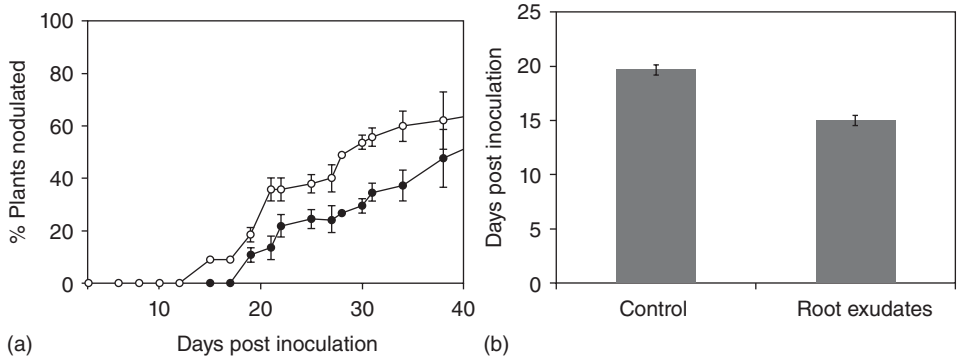

Figure 35.3 The effect of host root exudates pretreatment on plant nodulation frequency. Panel a shows a time course of plant nodulation. Prior to plant inoculation, *Frankia* CcI3 was pre-treated with *C. cunninghamiana* root exudates from nitrogen-deficient conditions (open circles) or 1/4 HS (N_2) medium (closed circles) for 6 days. The percentage of plants with nodules was determined as described in the "Methods" section. Data presented are the average percentage for three independent experiments. The average number of plants per condition in the three experiments was $n = 10$. Panel b shows the average number of days required to observe the first appearance of nodules on a plant in each treatment ($n = 3$). *Frankia* CcI3 was pretreated with *C. cunninghamiana* root exudates from nitrogen-deficient conditions or 1/4 HS (N_2) medium (Control) for 6 days. ANOVA analysis showed this to be a significant difference (p-value < 0.05). (From Beauchemin et al., 2012 with permission.)

Table 35.1 The effect of different plant root exudates on *Frankia* hyphal curling

Plant Root Exudates	*Frankia* sp. Strain CcI3	*Frankia* sp. Strain CN3	*Frankia* sp. Strain EUN1f	*Frankia alni* Strain ACN14a
Casuarina	+	–	–	–
Elaeagnus	–	–	+	+
Hippophae	–	–	+	–
Myrica	N.D.	–	–	+/–
Alnus	–	–	–	+
Betula	–	–	–	–
Hoagland Control	–	–	–	–

Symbols represent: +, hyphal curling; –, no curling, and +/–, an intermediate result.

Table 35.2 The effect of root exudates on the nodulation of actinorhizal plants

	Alnus Nodulation	
	1-Month Nodules per Plant	2-Month Nodules per Plant
ACN14a	4.0	7.6
ACN14a + *Alnus* root exudates	3.3	9.0
QA3	1.0	1.0
QA3 + *Alnus* root exudates	3.6	10.6
	Hippophae nodulation	
	1month	
EAN1pec	6.0	
EAN1pec + *Hippophae* root exudates	27.0	

The number of nodules per plant was counted after 1 month and 2 months.

Frankia strains for the hyphal curling response. Preliminary results are shown in Table 35.1. *Frankia* sp. strain CcI3 only responded to root exudates from *Casuarina. Frankia* sp. strain EUN1f responded toward root exudates from *Elaeagnus* and *Hippophae*, both host plant of this strain. *Frankia alni* strain ACN14a responded top *Alnus*, *Eleaegnus*, and *Myrica* root exudates, while the atypical *Frankia* sp. strain CN3 did not respond to any of the plant root exudates. The atypical *Frankia* strains are unable to reinfect their host plants and considered noninfective (Nod^-) and nonnitrogen-fixing (Fix^-) strains. These preliminary results indicate that the different host plants excrete a specific signal only perceived by their symbionts.

The effect of root exudates on plant infectivity was tested, and the preliminary results are presented in Table 35.2. The addition of *Alnus* root exudates increased the nodulation of *A. glutinosa* for both *Frankia* sp. strain QA3 and *Frankia alni* strain ACN14a. With the *Frankia* sp. strain EAN1pec, *Hippophae* root exudates increased the nodule formation of *H. rhamnoides.*

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Chapter 36

Exopolysaccharide Production in Rhizobia Is Regulated by Environmental Factors

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INTRODUCTION

Rhizobia comprise a very diverse group of soil bacteria that induce the formation of new specialized organs on roots and stems of leguminous plants, called nodules, inside of which atmospheric nitrogen is reduced to ammonia. The establishment of symbiosis is a complex process involving exchange of many signals between the symbiotic partners, with plant flavonoids and rhizobial lipochitin oligosaccharides being the most important molecules (Downie, 2010; see Chapters 50, 51). In addition, acidic extracellular polysaccharides (EPS) play a significant role in the early stages of symbiotic interactions, especially when the host plants form indeterminate-type nodules (e.g., *Medicago*, *Vicia*, and *Trifolium* spp.) (Janczarek, 2011). The ability to produce EPS is a widespread feature among rhizobia. This polysaccharide is weakly associated with the bacterial surface and released in large amounts into the environment. Several different functions are ascribed to EPS, for example, protection against stress factors and antimicrobial compounds, nutrient gathering, and attachment to both abiotic surfaces and plant roots (Downie, 2010). Moreover, this polymer is essential for biofilm formation, constituting the major component of its matrix. EPS-deficient mutants of *Sinorhizobium meliloti* and *Rhizobium leguminosarum* elicit formation of nonnitrogen-fixing nodules on their host plants (Borthakur et al., 1988; Glucksmann et al., 1993a, b; Rolfe et al., 1996; van Workum et al., 1997; Cheng and Walker, 1998; Janczarek et al., 1999). Although EPS is essential for a great majority of rhizobia that establish symbioses with leguminous plants forming indeterminate-type nodules, there are known examples of symbioses demonstrating some differences. *Sinorhizobium fredii* HH103 does not require EPS and KPS for the establishment of effective symbiosis with *Glycyrrhiza uralensis*, the host plant forming indeterminate-type nodules (Margaret-Oliver et al., 2012). On the other hand, alterations in EPS can affect the symbiosis of *Bradyrhizobium japonicum* USDA 110 with soybean, which forms determinate-type nodules (Quelas et al., 2010).

The synthesis of rhizobial EPS is a complex process regulated at both transcriptional and posttranscriptional levels and influenced by several nutritional and stress factors.

This review describes the genetic control of EPS synthesis in rhizobia and regulation of this process by various environmental signals.

36.1 CHEMICAL STRUCTURES OF RHIZOBIAL EXOPOLYSACCHARIDES

EPS produced by rhizobia are species- or even strain-specific heteropolymers consisting of repeating units that contain common monosaccharides: D-glucose, D-mannose, D-galactose, L-rhamnose, D-galacturonic and D-glucuronic acids, and noncarbohydrate residues such as acetyl, pyruvyl, and succinyl groups. The uronic acids and negatively charged pyruvyl groups contribute to the acidic character

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of this polysaccharide. Rhizobial EPS demonstrate high diversity in terms of the sugar composition, unit size, type of glycosidic linkages, and noncarbohydrate modifications. EPS are usually synthesized in two forms of different molecular masses, that is, high-molecular-weight (HMW) polymers of 10^6–10^7 Da and low-molecular-weight (LMW) forms consisting of monomers, dimers, and trimers of the repeating unit (González et al., 1998). The LMW EPS was found to be an active biological fraction required for successful infection of leguminous plants.

Fast-growing rhizobia (*Sinorhizobium* and *Rhizobium* species) produce EPS of lower diversity than those synthesized by slow-growing bacteria. A great majority of EPS of fast-growing rhizobia are composed of octasaccharide units, in which glucose is a dominant sugar component (Fig. 36.1). *S. meliloti* synthesizes two structurally distinct EPS: succinoglycan (EPS I) and galactoglucan (EPS II) produced under phosphate limitation (Her et al., 1990; Zhan et al., 1991; Reinhold et al., 1994). The repeating units of EPS I are composed of seven D-glucoses and one D-galactose joined by β-1,3, β-1,4, and β-1,6 glycosidic linkages, and substituted with acetyl, succinyl, and pyruvyl groups (Fig. 36.1a) (Reuber and Walker, 1993a,1993b; Reinhold et al., 1994; Zevenhuizen, 1997). In contrast, EPS II consists of disaccharide units that contain D-galactose and D-glucose joined by α-1,3 and β-1,3 linkages. All the galactosyl residues are substituted with 4,6-*O*-pyruvyl groups and most glucosyl residues are 6-*O*-acetylated (Fig. 36.1b) (Her et al., 1990; Reuber and Walker, 1993a,1993b; Zevenhuizen, 1997).

S. fredii NGR234 synthesizes EPS whose units contain D-glucose, D-galactose, and D-glucuronic acid in a molar ratio 4 : 2 : 2, modified with *O*-acetyl and pyruvyl groups (Fig. 36.1c) (Djordjevic et al., 1986).

The majority of strains belonging to three biovars of *R. leguminosarum* (*viciae, trifolii*, and *phaseoli*) secrete EPS containing identical octasaccharide units composed of D-glucose, D-glucuronic acid, and D-galactose residues in a molar ratio 5 : 2 : 1 (Fig. 36.1d) (Robertsen et al., 1981; McNeil et al., 1986; O'Neill et al., 1991; Philip-Hollingsworth et al., 1989; Orgambide et al., 1992; Lopez-Lara et al., 1993; Breedveld et al., 1993a, b). The backbone of the units contains two glucose and two glucuronic acid residues joined by α-1,4 and β-1,4 glycosidic linkages. However, several *R. leguminosarum* strains produce EPS whose units vary in the length of the side chain, patterns of noncarbohydrate modifications, and the type of linkages (McNeil et al., 1986; Canter Cremers et al., 1991). For example, the units of *R. leguminosarum* bv. *viciae* 248 EPS have an extra glucuronic acid residue in the side chain, whereas the subunits of the *R. leguminosarum* bv. *trifolii* 4S EPS do not contain terminal galactoses (Amemura et al., 1983; Canter Cremers et al., 1991). For more details, see (Ivashina and Ksenzenko, 2012). The units of *R. leguminosarum* EPS are usually substituted by two pyruvyl, one or two nonstoichiometric *O*-acetyl, and one nonstoichiometric 3-hydroxybutanoyl groups. However, the distribution pattern of *O*-acetyl and hydroxybutanoyl modifications is dependent on the culture medium and bacterial growth phase and may vary for some *R. leguminosarum* strains (McNeil et al., 1986; Canter Cremers et al., 1991).

Rhizobium tropici also produces EPS composed of octasaccharide units, which contain D-glucose and D-galactose residues in a molar ratio 6 : 2, substituted with *O*-acetyl and pyruvyl groups (Fig. 36.1e) (Gil-Serrano et al., 1990).

EPS produced by slow-growing bradyrhizobia show even higher diversity in comparison to those synthesized by fast-growing rhizobia, which mainly involves the sugar composition and size of the units. In addition, unit modification by *O*-methyl groups seems to be unique to this group of rhizobia. EPS of *Bradyrhizobium japonicum* USDA 110 displays the highest structural diversity, since it is composed of pentasaccharide units containing four different sugars: D-glucose, D-mannose, D-galacturonic acid, and D-galactose (a molar ratio 2 : 1 : 1 : 1) substituted with *O*-methyl and *O*-acetyl groups (Fig. 36.1f) (Minamisawa, 1989; Poveda et al., 1997). Moreover, the structure of NPS, the polysaccharide produced by *B. japonicum* cells inside root nodules, significantly differs from that of EPS synthesized by free-living bacteria (Fig. 36.1g) (An et al., 1995; Streeter et al., 1992). In contrast, *B. elkani* produces EPS composed of tetrasaccharide units that contain only two sugars, L-rhamnose and D-glucuronic acid, in a molar ratio 3 : 1 (Fig. 36.1h) (An et al., 1995). In addition, both EPS and NPS produced by *B. elkani* have an identical structure.

An interesting example of an exopolysaccharide whose structure essentially differs from that of all the above-described EPS is a polymer secreted by *Azorhizobium caulinodans* – the microsymbiont of *Sesbania rostrata*. It is a linear homopolysaccharide consisting of 4,6-*O*-pyruvyl-D-galactosyl residues linked with α-1,3 bonds (D'Haeze et al., 2004).

36.2 GENETIC CONTROL OF EPS SYNTHESIS

The biosynthesis of heteropolysaccharides in rhizobia is a multistep process, requiring the activity of many different enzymes involved in the synthesis of nucleotide sugar precursors and noncarbohydrate donors, assembly, and modification of repeating units, as well as polymerization and transport of EPS outside bacteria (Glucksmann et al., 1993a,1993b; Whitfield, 1995). EPS synthesis is carried out by a multienzymatic complex located in both bacterial inner (IM) and outer (OM) membranes. Polyprenyl lipid

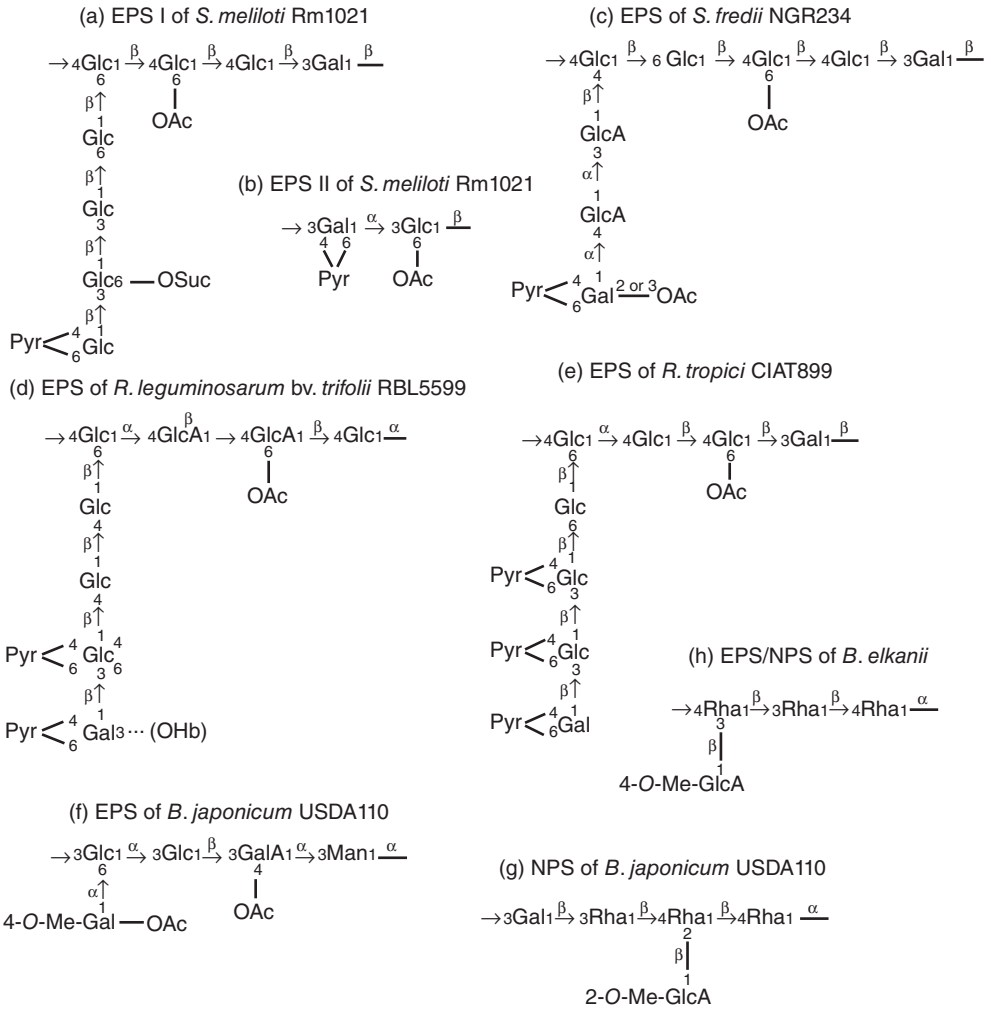

Figure 36.1 The chemical structures of EPS repeating units from different rhizobial species: (a) *S. meliloti* EPS I (Reinhold et al., 1994; Zevenhuizen, 1997; Reuber and Walker, 1993a,1993b); (b) *S. meliloti* EPS II (Zhan et al., 1991; Zevenhuizen, 1997; Her et al., 1990); (c) *S. fredii* NGR234 (Djordjevic et al., 1986); (d) *R. leguminosarum* bv. *trifolii* (Robertsen et al., 1981; O'Neill et al., 1991; Breedveld et al., 1993a); (e) *R. tropici* (Gil-Serrano et al., 1990); (f) *B. japonicum* (Minamisawa, 1989; Poveda et al., 1997); (g) NPS of *B. japonicum* (Streeter et al., 1992; An et al., 1995); (h) *B. elkanii* (An et al., 1995). Abbreviations: Glc, glucose; Gal, galactose; Man, mannose; Rha, rhamnose; GlcA, glucuronic acid; GalA, galacturonic acid; OAc, *O*-acetyl; Pyr, ketal pyruvate; Suc, succinyl; Me, methyl groups.

carriers anchored in the inner leaflet of the IM are acceptors to which nucleotide diphosphosugar precursors are sequentially bonded by specific glycosyltransferases. Then, assembled units are flipped across the IM to the periplasmic space by the Wzx-like translocase (Whitfield and Paiment, 2003; Liu et al., 1996). Polymerization of the units is most probably coupled with secretion of EPS outside bacteria. In this process, both the Wzy-like polymerase and the Wzc-like IM-periplasmic auxiliary protein are engaged (Paulsen et al., 1997; Whitfield and Paiment, 2003).

Genes involved in the synthesis of rhizobial EPS are usually grouped in large clusters located on chromosomes or megaplasmids (Fig. 36.2) (Kaneko et al., 2000; Finan et al., 2001; Kaneko et al., 2002; Young et al., 2006; González et al., 2006; Król et al., 2007; Reeve et al., 2010a, b). Such organization probably reflects coordinated and tightly regulated expression of EPS synthesis genes.

36.2.1 Genes Involved in the Synthesis of Succinoglycan and Galactoglucan in *S. meliloti*

So far, EPS synthesis and regulation of this process in rhizobia have been most extensively studied in *S. meliloti*. In this species, genes involved in succinoglycan synthesis are located on the pSymB megaplasmid in a large cluster

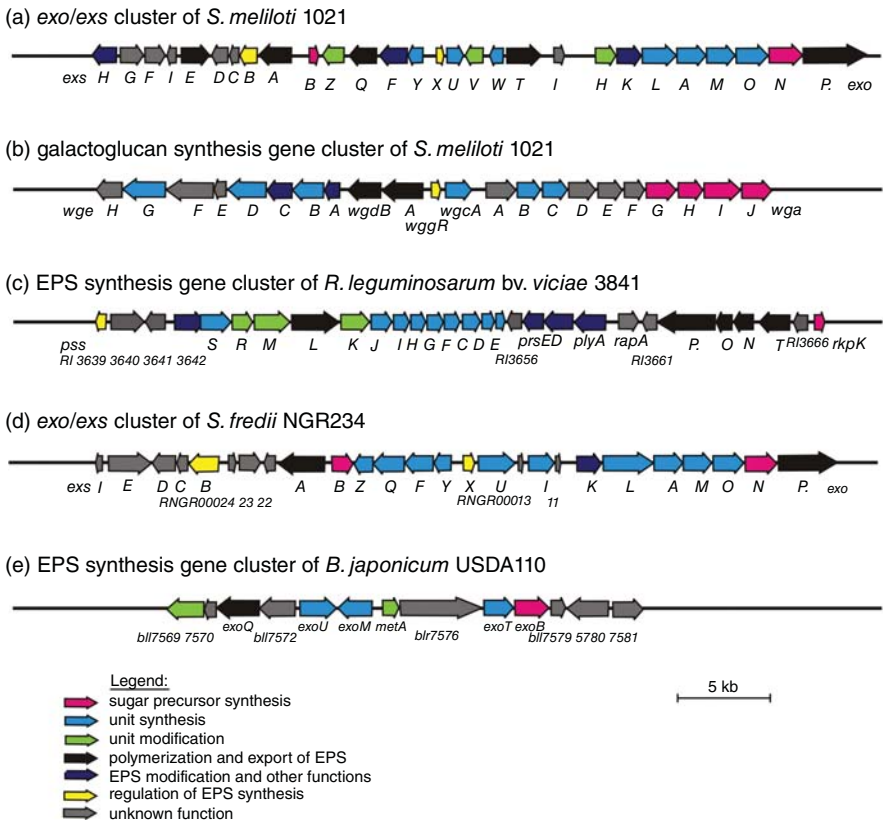

Figure 36.2 Genetic organization of the clusters involved in exopolysaccharide synthesis in several rhizobial species: (a) the *exo/exs* cluster of *S. meliloti* (Finan et al., 2001); (b) the galactoglucan synthesis gene cluster of *S. meliloti* (Finan et al., 2001); (c) *R. leguminosarum* bv. *viciae* 3841 cluster (Young et al., 2006); (d) *S. fredii* NGR234 cluster (Acc. no. AY316746); (e) *B. japonicum* USDA 110 cluster (Kaneko et al., 2002) (the figure is the modified figure from the reference (Janczarek, 2011)).

(~35 kb) containing 28 *exo/exs* genes (Fig. 36.2a) (Reuber and Walker, 1993b; Finan et al., 2001; Glucksmann et al., 1993a,1993b; Becker et al., 1993a,1993b,1993c). This cluster contains genes encoding enzymes responsible for the synthesis of nucleotide sugar precursors (*exoN* and *exoB*), unit assembly (*exoY*, *exoF*, *exoA*, *exoL*, *exoM*, *exoO*, *exoU*, and *exoW*) and modification (*exoH*, *exoV*, and *exoZ*), and polymerization and transport of EPS (*exoP*, *exoQ*, *exoT*, and *exsA*) (Glucksmann et al., 1993a,1993b; Becker et al., 1993a,1993b,1993c; Müller et al., 1993; Becker et al., 1995a,1995b). In addition, genes involved in regulation of EPS I (*exoR*, *exoS*, *exoD*, and *mucR*) and sugar precursor synthesis (*exoC*) are dispersed throughout the chromosome (Doherty et al., 1988; Uttaro et al., 1990; Reed et al., 1991b; Reed and Walker, 1991; Keller et al., 1995).

The assembly of octasaccharide units of EPS I is initiated by the addition of UDP-galactose to the lipid carrier located in the IM. In this step, two galactosyl-IP-transferase ExoY and auxiliary ExoF proteins are involved (Müller et al., 1993). The subsequent steps of unit assembly are carried out by glucosyltransferases encoded by *exoALMOUW* genes (Reuber and Walker, 1993a,1993b; Glucksmann et al., 1993b; Becker et al., 1993b,1993c). However, the enzyme responsible for the addition of the last sugar residue to the units has not yet been identified. *S. meliloti* strains carrying mutations in the *exoY*, *exoA*, *exoL*, and *exoM* genes do not produce EPS I and form nodules inefficient in nitrogen fixation (Glucksmann et al., 1993b; Müller et al., 1993; Becker et al., 1993b; Cheng and Walker, 1998).

Three enzymes are responsible for nonsugar modifications of EPS I: ExoV is required for the addition of pyruvyl; ExoZ modifies with acetyl; and ExoH provides succinyl groups to the units (Fig. 36.2a). The presence of succinyl groups in the EPS I is indispensable for the formation of the LMW fraction of this polysaccharide. The importance of this modification is confirmed by the phenotype of the *exoH* mutant, which produces exclusively HMW EPS I lacking succinyl groups and elicits ineffective nodules on alfalfa (Cheng and Walker, 1998). The *S. meliloti* strain carrying a mutation in *exoV* synthesizes only monomer units, indicating that this modification of the units is crucial for polymerization of succinoglycan (Reuber and Walker, 1993b; Becker et al., 1993c; York and Walker, 1998). In contrast, the *exoZ* mutant exhibits only a slightly diminished efficiency in infection thread formation and induces nitrogen-fixing nodules on its host plant (Reuber and Walker, 1993a; Buendia et al., 1991). Moreover, the presence of succinyl and acetyl substituents in EPS I affects the susceptibility of this polymer to cleavage by two glycanases (β-1,3-1,4-glucanase ExoK and succinoglycan depolymerase ExsH) (York and Walker, 1998).

Polymerization and secretion of EPS I are carried out by ExoP, ExoQ, and ExoT proteins (Fig. 36.2a) (Glucksmann et al., 1993a; Becker et al., 1993b). ExoP is an autophosphorylated tyrosine kinase. The N-terminal domain of this protein located mainly in the periplasmic space is crucial for EPS I polymerization, whereas the C-terminal domain, displaying ATPase activity, has a regulatory function (Becker et al., 1995b; Jofre and Becker, 2009). In addition, ExoQ is responsible for the production of HMW EPS I, whereas ExoT is indispensable for the synthesis of its LMW forms (trimers and tetramers). Also, the ExsA protein was found to be required for the export of HMW EPS I (Becker et al., 1995a).

The biosynthesis of EPS II is directed by genes grouped in a 27-kb cluster on the pSymB plasmid (Fig. 36.2b) (Becker et al., 1997; Moreira et al., 2000). In this cluster, 22 genes organized in five *wga*, *wgc*, *wggR*, *wgd*, and *wge* operons have been identified. The *wgaG*, *wgaH*, *wgaI*, and *wgaJ* genes are involved in the synthesis of deoxythymidine diphosphosugar precursors (dTDP-glucose and dTDP-rhamnose). The WgaB and WgeB proteins are putative β-glucosyltransferases, whereas the WgaC, WgcA, WgeD, and WgeG proteins are galactosyltransferases. Other genes of this cluster code for proteins potentially engaged in polymerization (*wgdA* and *wgdB*) and regulation of EPS II synthesis (*wggR*) (Becker et al., 1998; Moreira et al., 2000; Bahlawane et al., 2008).

36.2.2 Genes Involved in the Synthesis of EPS in *R. leguminosarum*

In the case of *R. leguminosarum*, the data concerning EPS synthesis are much more fragmentary. So far, precise functions in this process have been experimentally established only for a few proteins. The functions of other gene products have been predicted based on their sequence similarities to proteins deposited in databases. A majority of genes involved in the synthesis of EPS are grouped in a 35-kb chromosomal cluster, called Pss-I (Fig. 36.2c) (Young et al., 2006; Król et al., 2007). This region is highly conserved among all the hitherto sequenced genomes of *R. leguminosarum* and *R. etli* strains (González et al., 2006; Reeve et al., 2010a,2010b). Only some slight differences in nucleotide sequences and gene contents have been noticed between these strains (Ivashina and Ksenzenko, 2012). The Pss-I cluster encompasses genes required for the unit synthesis (*pssEDCFGHIJS*) and modification (*pssKMR*), polymerization, and export of EPS (*pssLTNOP*), as well as EPS processing (*pssW, plyA*, and *prsDE*). *exo5* (*rkpK*) is also located in the Pss-I cluster and together with the *exoB* gene, which is not linked to this region, is involved in the synthesis of nucleotide sugar precursors (UDP-glucuronic acid and UDP-galactose, respectively). The *exo5* gene encodes UDP-glucose dehydrogenase, which converts UDP-glucose to UDP-glucuronic acid. The mutant in this gene does not produce either extracellular or capsular polysaccharides and synthesizes lipopolysaccharide lacking galacturonic acid (Laus et al., 2004; Muszyński et al., 2011). UDP-galactose synthesized by ExoB (a UDP-glucose 4-epimerase) is a sugar precursor for the production of EPS and other galactose-containing polysaccharides (Canter Cremers et al., 1990). The *exoB* mutant produces EPS that does not contain terminal galactoses in its units, and this structural alteration strongly affects the ability of these bacteria to invade host plant roots (Sánchez-Andújar et al., 1997).

The assembly of EPS units is initiated by the PssA protein, which transfers glucose-1-phosphate from UDP-glucose to the lipid carrier. *pssA* encoding this enzyme is located on the chromosome at a long distance from the Pss-I cluster and is transcribed as a monocistronic mRNA (Borthakur et al., 1988; Ivashina et al., 1994; Janczarek et al., 1999). This gene is highly conserved in *R. leguminosarum* strains and the closely relatives *R. etli* and *R. gallicum* (Janczarek and Skorupska, 2003; Janczarek et al., 2009b). *pssA* mutants of *R. leguminosarum* do not produce EPS and induce nonnitrogen-fixing nodules on their host plants (clover, pea, and vetch) (Ivashina et al., 1994; Rolfe et al., 1996; van Workum et al., 1997; Janczarek et al., 1999).

The subsequent steps of the unit assembly are carried out by enzymes encoded by genes located in the Pss-I cluster (Fig. 36.2c). Among them, *pssD* and *pssE* genes encode glucuronosyl-β-1,4-glucosyltransferase, which catalyses the addition of the second sugar to glucose attached to the lipid carrier (Pollock et al., 1998). The *pssD* mutant displays a very similar phenotype to the *pssA* mutant; it does not produce EPS and elicits ineffective nodules on host roots. The third step of the unit synthesis is conducted by glucuronosyl-β-1,4-glucuronosyltransferase PssC (van Workum et al., 1997; Pollock et al., 1998). A mutation in *pssC* results in a twofold decrease in EPS synthesis and failed nodulation of vetch (Sadykov et al., 1998).

The *exo-344* (*pssJ*) gene encoding galactosyltransferase is most probably involved in the last step of the unit assembly. Breedveld et al. (1993a) described an *exo-344*::Tn*5* mutant of *R. leguminosarum*, which produced only heptasaccharide units lacking the terminal galactose.

Up to now, enzymes responsible for the remaining steps of the unit synthesis have not been characterized experimentally, although some Pss proteins encoded by the Pss-I cluster are most probably engaged in this process (Fig. 36.2c) (Sadykov et al., 1998; Król et al., 2007). Recently, Ivashina and Ksenzenko (2012) have proposed a model of the synthesis of the EPS units in *R. leguminosarum* based on sequence similarities of these proteins to enzymes available in databases and phenotypes of a few *pss* mutants. They postulate that the subsequent steps of the unit assembly are carried out by the PssS, PssF, PssI/PssG, and PssH/PssI

glycosyltransferases, respectively. Mutations introduced into the *pssFGHI* genes of *R. leguminosarum* bv. *viciae* VF39 only result in a decrease in EPS production, but they do not affect the structure of the synthesized polymer. Other genes of the Pss-I cluster, *pssR* encoding acetyltransfease, *pssM* encoding ketal pyruvate transferase, and *pssK* encoding for pyruvyltransferase, are involved in nonsugar modifications of the units. A mutation in *pssR* causes a significant reduction of acetyl groups in EPS, but this alteration in its composition has no effect on nodule development and nitrogen fixation (Ivashina and Ksenzenko, 2012). Moreover, two enzymes are responsible for unit pyruvylation; PssM is needed for the addition of the pyruvyl group to the subterminal glucose, whereas PssK is indispensable for pyruvylation of the terminal galactose. Bacteria carrying *pssM* mutation exhibit impaired differentiation into bacteroids and elicit nonnitrogen-fixing nodules on peas (Ivashina et al., 2010). A mutation in *pssK* exerts a stronger negative effect, since bacteria having a Tn*5* insertion in this gene do not produce EPS and are defective in nitrogen fixation (Ivashina and Ksenzenko, 2012). The above data indicate that this type of unit modification is essential for EPS polymerization in *R. leguminosarum*, as in the case of *S. meliloti* EPS I (Becker et al., 1993c; Glucksmann et al., 1993a).

Polymerization and export of EPS are carried out by a Wzx/Wzy-type secretion system consisting of proteins encoded by *pssL* and *pssTNOP* genes (Fig. 36.2c). The PssL protein displays significant similarity to Wzx-type flippases, which participate in the O-antigen translocation from the inner to the outer leaflet of the IM, whereas PssT is an integral IM protein showing similarity to Wzy-like proteins (Mazur et al., 2003; 2005). The PssP protein displays homology to *S. meliloti* ExoP and other membrane-periplasmic auxiliary proteins involved in polysaccharide synthesis (Mazur et al., 2002). A deletion of *pssP* abolishes EPS synthesis in *R. leguminosarum*, whereas a mutation in *pssT* results in overproduction of this polymer. PssN is a lipoprotein associated with the OM, which interacts with both the PssP copolymerase and the PssO protein located in the OM (Marczak et al., 2006; 2012). A *pssO* mutation abolishes EPS synthesis and negatively affects nitrogen fixation (Marczak et al., 2008).

The Pss-I cluster also contains genes involved in EPS processing (Fig. 36.2c). These include *prsDE* genes encoding components of the type I secretion system and *plyA* encoding glycosyl hydrolase (Finnie et al., 1997; 1998; Krehenbrink and Downie, 2008). The PlyA together with PlyB glycanase have the ability to cleave EPS and affect its processing (Zorreguieta et al., 2000). A *prsD* mutant synthesizes EPS of a higher degree of polymerization than the wild-type strain and induces formation of ineffective nodules on its host plant (Finnie et al., 1997).

36.2.3 Genes Involved in EPS Synthesis in Other Rhizobial Species

A region containing *exo* genes organized in several operons has been identified in the pNGR234b plasmid of *S. fredii* NGR234 (Fig. 36.2d) (Gray et al., 1990; Zhan et al., 1990). Large fragments of the *S. fredii exo* cluster are closely related or even identical to the *S. meliloti exo* region (these concern the *exoABYLMNPX* genes). The presence of these *exo* homologs in both the *S. fredii* NGR234 and *S. meliloti* genomes can be explained by very similar structures of EPS produced by these bacterial species. However, some differences in these clusters have been found (Zhan et al., 1990; Staehelin et al., 2006). These concern *exoV*, *exoW*, *exoT*, and *exoH* genes, which are absent in the *exo* cluster of *S. fredii* NGR234 (e.g., *exoH* is not located in the pNGR234b plasmid but in the genome) (Schmeisser et al., 2009). In *S. meliloti*, ExoH is needed for the addition of succinyl groups to the units of EPS I, whereas EPS of *S. fredii* NGR234 is not succinylated.

Similarly, a region involved in EPS synthesis has been identified in the genome of *B. japonicum* USDA 110 (Fig. 36.2e) (Becker et al., 1998; Kaneko et al., 2002). This cluster contains several genes organized in a few operons, among them genes encoding homologs of *S. meliloti* ExoB, glycosyltransferases, and ExoP. A mutant with a deletion of the genomic fragment encoding the C-terminal domain of ExoP, the entire ExoT, and the N-terminal domain of ExoB, produces only the LMW form of EPS lacking galactose and nodulates the host plant with a delay, inducing symptoms of plant defense reactions (Becker et al., 1998).

The data concerning EPS synthesis in moderately growing *Mesorhizobium* are also scarce. Recently, a region involved in the synthesis of this heteropolymer has been identified in the genome of *M. tianshanense* (Wang et al., 2008). In this cluster, *mtpABCD* and *mtpE* genes were found, which display significant similarity to the *R. leguminosarum pssTNOP* and *exo5* genes, respectively. Mutants in both *mtpABCD* and *mtpE* operons do not produce EPS and are defective in nodulation of their host plant (*Glycyrrhiza uralensis*) (Wang et al., 2008).

36.3 REGULATION OF EPS SYNTHESIS IN RHIZOBIA

EPS synthesis in rhizobia, a complex process regulated at different molecular levels, has been the most extensively studied in *S. meliloti* to date. Several environmental factors and stress conditions influence EPS production, affecting the amount and/or composition of the synthesized polymer (Zevenhuizen, 1997; Breedveld et al., 1993b; Quelas et al., 2006; Bardin and Finan, 1998; Mendrygal and González, 2000; Janczarek and Skorupska, 2009; 2011).

36.3.1 Regulation of EPS Synthesis in *S. meliloti*

36.3.1.1 Regulation of EPS I Synthesis. Depending on the culture conditions, *S. meliloti* produces two distinct EPS: succinoglycan and galactoglucan. To date, several genes involved in the regulation of the synthesis of these polymers have been identified on both the chromosome (*exoR*, *exoS*, *exoD, mucR*, *expR*, *syrM*, and *phoB*) and on the pSymB megaplasmid (*exoX*, *exsB*, and *wggR*). A great majority of these genes encode repressors, whereas the two *syrM* and *phoB* genes code for positive regulators of EPS I and EPS II synthesis, respectively (Reed et al., 1991a,1991b; Becker et al., 1995a; Keller et al., 1995; Rüberg et al., 1999; Mendrygal and González, 2000). A protein encoded by *mucR* is the main regulator of these biosynthetic pathways, acting as a positive regulator of EPS I synthesis and a negative regulator of EPS II synthesis (Rüberg et al., 1999; Bertram-Drogatz et al., 1998).

In general, regulation of succinoglycan production is almost always negative (Fig. 36.3). Up to now, six genes have been found to affect this process negatively. These include the *exoR*, *exoS*, *cbrA*, and *emmC* genes located on the chromosome and the *exoX* and *exsB* genes located in the *exo/exs* cluster. Mutations in both *exoS* and *exoR* genes result in a higher level of expression of several *exo* genes and increased production of EPS I in comparison to the wild-type strain (Doherty et al., 1988; Reed et al., 1991b). ExoS is a sensor protein of a two-component ExoS/ChvI regulatory system responsible for the recognition of environmental signals (Osterås et al., 1995). This protein with kinase activity is located in the IM and its sensor domain is directed to the periplasmic space. ExoS activates ChvI by phosphorylation, and this protein subsequently affects the transcription of several *exo* genes (Fig. 36.3). ExoR is located in the periplasmic space, where it interacts with the sensor domain of the ExoS protein and inhibits the ExoS/ChvI system signaling (Cheng and Walker, 1998; Wells et al., 2007; Chen et al., 2008). The ExoR protein negatively regulates the transcription of its own gene. In this regulation, the ExoS/ChvI system is also engaged, which positively affects *exoR* expression (Chen et al., 2008; Lu and Cheng, 2010). Phenotypic analyses of *exoS* and *chvI* mutants have indicated that this regulatory system is additionally required for growth on different carbon sources (Bélanger et al., 2009; Wang et al., 2010). Moreover, the *chvI* mutant exhibits hypermotility, lower tolerance to acidic conditions, and a lower level of poly-3-hydroxybutyrate synthesis than the wild-type strain. These data indicate that the ExoR, ExoS, and ChvI proteins form a regulatory system involved in several cell processes, including EPS I synthesis, nutrient utilization, and motility.

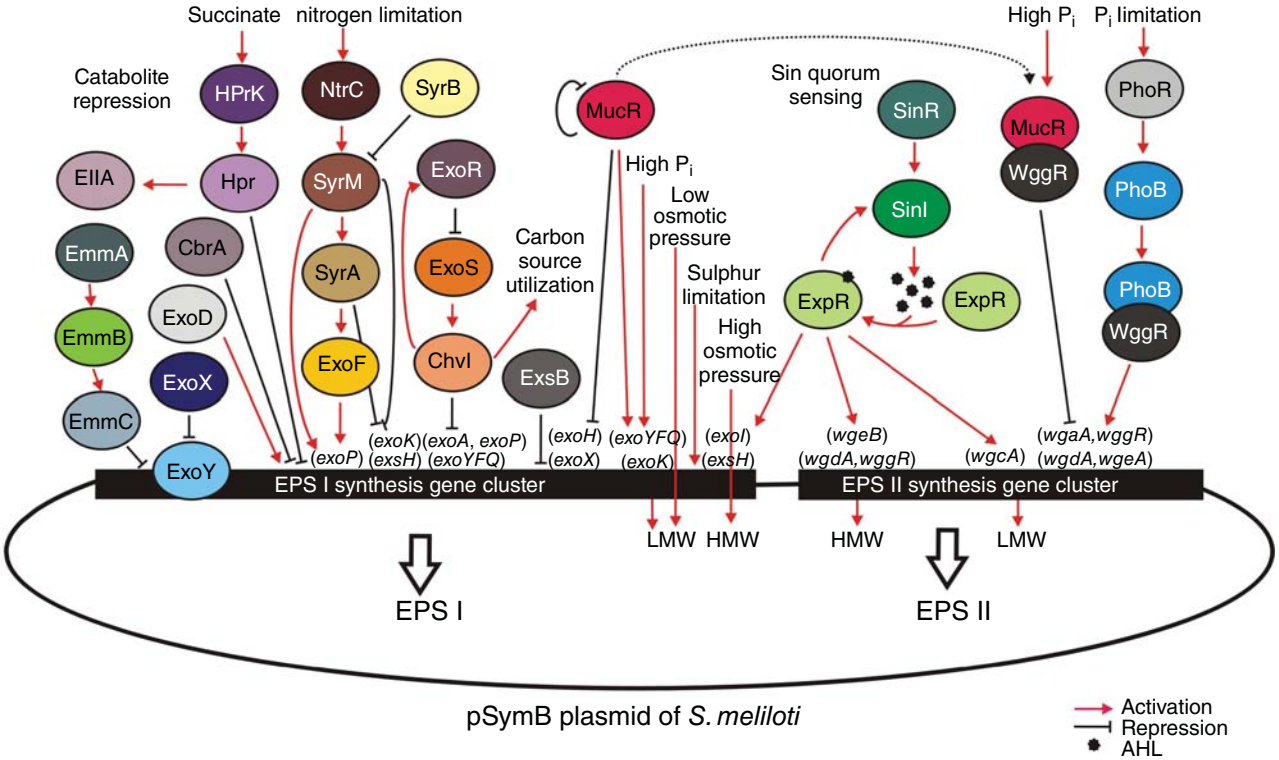

Figure 36.3 Regulation of EPS I and EPS II syntheses in *S. meliloti* (the figure is a modified version of the figure from the reference (Janczarek, 2011)).

exoX is another gene of the *exo/exs* cluster, which negatively regulates the synthesis of EPS I (Fig. 36.3). It has been found that the ratio of *exoX* to *exoY* copies is very important for the proper level of the synthesis of this polymer. ExoX is a small, inner membrane protein showing significant similarity to PsiA of *R. leguminosarum* bv. *phaseoli* and ExoX of *S. fredii* NGR234 (Borthakur et al., 1988; Gray et al., 1990). An *exoX* mutant overproduces EPS I, whereas a strain carrying multiple copies of this gene does not synthesize this polymer. It has been suggested that ExoX functions as a posttranscriptional inhibitor of the ExoY protein, since *exoX* does not affect the expression of *exo* genes (Müller et al., 1993; Reed et al., 1991a). The same localization of the ExoX and ExoY proteins makes their direct interaction possible.

Similarly, *exsB* is involved in negative regulation of succinoglycan synthesis (Fig. 36.3) (Becker et al., 1995a). A mutant in this gene produces threefold more EPS I than the wild-type strain, whereas a strain carrying additional *exsB* copies synthesizes decreased amounts of the polysaccharide. Since the transcription of *exo* genes in this mutant does not differ in relation to the wild type (with the exception of *exoK*), it has been proposed that ExsB acts mainly at the posttranscriptional level (Becker et al., 1993b; Becker et al., 1995a).

Recently, a novel EmmA/EmmB/EmmC system has been identified in *S. meliloti*, which plays an important role in regulation of several cell processes including EPS I production, motility, and stress adaptation (Morris and González, 2009). This three-component system shows some functional similarity to the ExoR/ExoS/ChvI system described earlier. EmmA is a periplasmic protein, whereas EmmB and EmmC are sensor and response proteins, respectively. Mutations in the *emm* genes result in increased production of succinoglycan, decreased motility, and stress tolerance, and as a consequence, ineffective symbiosis with alfalfa. However, *exo* genes regulated by this system have not been established.

Also, *cbrA* coding for a stationary phase-induced sensor kinase negatively affects succinoglycan synthesis (Gibson et al., 2007). A mutant in this gene overproduces EPS I and demonstrates several cell envelope and symbiotic defects.

Among the regulators, MucR seems to be a main protein involved in positive regulation of succinoglycan synthesis, since the *mucR* mutant produces only residual amounts of the LMW form of this polysaccharide (Keller et al., 1995). MucR containing a C_2H_2 type zinc-finger motif binds to a palindromic sequence, called the MucR-box, in the *mucR* upstream region and negatively regulates the transcription of its own gene. Moreover, this protein binds to promoter regions of some *exo* genes and modulates their expression. It has been established that the MucR protein functions as the activator of expression of *exoK* and *exoYFQ* genes and the repressor decreasing *exoH* and *exoX* transcription (Bertram-Drogatz et al., 1997; 1998).

Likewise, *exoD* seems to be involved in positive regulation of EPS I synthesis, although its precise role in this process has not been established. A mutant in this gene produces diminished amounts of EPS I and is sensitive to alkaline conditions (Reed and Walker, 1991).

The synthesis of *S. meliloti* exopolysaccharides is affected by several nutritional and stress conditions. Limitations of nitrogen and sulfur and high concentrations of phosphate stimulate production of EPS I (Fig. 36.3) (Doherty et al., 1988). On the other hand, phosphate starvation induces EPS II synthesis, indicating that this nutrient is an important signal determining which of these biosynthetic pathways will be activated (Zhan et al., 1991; Rüberg et al., 1999; Mendrygal and González, 2000). Moreover, osmotic conditions affect EPS synthesis in *S. meliloti*. A low osmotic pressure results in production of LMW EPS I, whereas an increased osmotic pressure stimulates the synthesis of the HMW form of this polymer (Breedveld et al., 1990).

Additionally, nitrogen significantly affects the synthesis of succinoglycan, and the NtrC and SyrM proteins act as positive regulators of this process. Mutations in both *ntrC* and *syrM* genes result in a decrease of EPS I production under nitrogen starvation. NtrC is at the top of this regulatory cascade and induces *syrM* expression due to a deficiency of this nutrient (Dusha et al., 1999). Subsequently, SyrM activates the transcription of *syrA* and *exoP*, but represses the *exoK* and *exsH* genes. Similarly, the SyrA protein decreases the transcription of *exoK* and *exsH* genes, whereas it increases *exoF* expression (Dusha et al., 1999; Barnett et al., 1998). SyrM displays significant similarity to NodD regulators responsible for activation of the expression of nodulation genes (Barnett and Long, 1990). It has been confirmed that this protein not only influences EPS I synthesis but is also involved in determination of the ratio of its LMW to HMW forms. In addition, the SyrB protein encoded by a gene located on the pSymA plasmid negatively regulates *syrM* expression (Fig. 36.3) (Barnett and Long, 1997).

Moreover, a phosphotransferase system (PTS; see Chapter 34) involved in succinate-mediated catabolite repression in *S. meliloti* negatively influences EPS I production (Pinedo et al., 2008; Pinedo and Gage, 2009). A chromosomal cluster located downstream of the *exoS* contains *hprK*, *EIIA*, and *hpr* genes encoding components of the PTS system. Succinate is a preferred carbon source playing a special role in the metabolism of this bacterium. In the presence of succinate, the HPrK protein activates HPr and the phosphorylated form of this protein subsequently activates EIIA, leading to catabolite repression of regulated genes. Probably, the expression of some *exo/exs* genes is affected by the PTS system, since mutants in both *EIIA* and *hprK* genes produce significantly more EPS I than the wild-type strain (Pinedo et al., 2008; Pinedo and Gage, 2009).

36.3.1.2 Regulation of EPS II synthesis. Phosphate is the most essential environmental factor in stimulation of EPS II production. In contrast to succinoglycan, which is synthesized in the presence of high concentrations of phosphate (>10 mM), low concentrations of this nutrient usually found in soil (<10 μM), induce production of galactoglucan (Zhan et al., 1991; Mendrygal and González, 2000). This polymer most probably plays a dominant role in natural soil conditions and its LMW form is crucial for both root colonization and biofilm formation (Rinaudi and González, 2009).

Phosphate regulates many genes of *S. meliloti*, including those involved in EPS II synthesis (Rüberg et al., 1999; Krol and Becker, 2004). A two-component regulatory system consisting of the sensor protein PhoR and the response regulator PhoB is engaged in this regulation (Fig. 36.3). Under P_i limitation, the PhoR kinase activates PhoB, which binds to PHO-boxes located in the promoters of phosphate-regulated genes and induces their transcription (Yuan et al., 2006). It has been confirmed that several galactoglucan synthesis genes (*wgaA*, *wggR*, *wgdA*, and *wgeA*) belong to the Pho regulon and their expression significantly increases under P_i starvation (Krol and Becker, 2004).

In addition, two other regulatory proteins, WggR (ExpG) and MucR, participate in the regulation of EPS II synthesis (Becker et al., 1997; Bahlawane et al., 2008). Depending on the phosphate concentration, the WggR protein functions as an activator or a repressor of the expression of galactoglucan synthesis genes. Under P_i starvation, this protein binds to palindromic sequences in the promoter regions of the *wggR*, *wgdA*, *wgeA*, and *wgaA* genes and activates their transcription (Rüberg et al., 1999; Bahlawane et al., 2008). Under the same conditions, a mutation in *wggR* causes a decrease in EPS II production. In contrast, WggR together with MucR represses the expression of galactoglucan synthesis genes in the presence of high phosphate.

The transcription of the *wga*, *wge*, and *wgd* operons is directed by two distal and proximal promoters (Bahlawane et al., 2008). Motifs recognized by the MucR, WggR, and PhoB proteins have been identified upstream of the distal promoters of these genes. Under P_i starvation, the activated PhoB protein in cooperation with WggR binds to the promoter regions of the *wga*, *wgd*, and *wge* genes and induces their transcription. In contrast, under high-phosphate conditions, the transcription of these genes is strongly repressed by MucR, which binds to MucR-boxes located close to the binding sites of PhoB and WggR and inhibits their activity (Baumgarth et al., 2005; Bahlawane et al., 2008). These data indicate that the cooperative action of the WggR, PhoB, and MucR proteins ensures fine-tuning of the expression of the galactoglucan synthesis genes, which enables adaptation of *S. meliloti* to changing environmental conditions.

36.3.1.3 The Influence of Quorum Sensing on the Synthesis of S. meliloti Exopolysaccharides. In *S. meliloti*, a quorum-sensing system Sin regulates the expression of many genes, among them those involved in the synthesis of EPS I and EPS II, motility, chemotaxis, and nitrogen fixation (see Chapter 37). This system consists of a LuxR-type transcriptional regulator SinR and an autoinducer synthase SinI responsible for the synthesis of long-chain *N*-acyl homoserine lactones (AHL), which are released outside cells and whose concentration serves as a signal of bacterial population density (Marketon et al., 2003). The regulation of a majority of these genes requires the presence of an additional LuxR-type regulator ExpR (Hoang et al., 2004). The expression of *sinI* is regulated by the SinR and ExpR proteins (Fig. 36.3). SinR activates *sinI* transcription and, subsequently, SinI synthesizes AHL. In addition, the ExpR protein activated by AHL positively affects the transcription of *sinI*.

The *S. meliloti* wild-type strain produces large amounts of galactoglucan, which is responsible for the mucoid phenotype of colonies. In contrast, both *sinI* and *expR* mutants are defective in EPS II production and form dry colonies on agar plates, confirming the importance of the Sin system and the ExpR protein in the synthesis of this polymer (Hoang et al., 2004; McIntosh et al., 2008). It has been shown that the transcription of galactoglucan synthesis genes (particularly *wgcA*, *wgdA*, *wgeB*, and *wggR*) is highly induced by ExpR in the presence of AHL (Marketon et al., 2003; Pellock et al., 2002). Moreover, the expression of the *wge*, *wga*, and *wgd* operons is required for the synthesis of HMW EPS II, whereas *wgcA* expression is critical for the synthesis of its LMW form. Recently, it has been confirmed that the ExpR protein activated by AHL directly binds to promoter regions of the *sinI*, *wgaA*, and *wgeA* genes (McIntosh et al., 2008). Activation of transcription of these genes by ExpR is dependent on another regulator WggR. This protein most probably functions as a mediator in regulation of the galactoglucan synthesis genes under different conditions, which coordinates the action of the ExpR, PhoB, and MucR regulators (Bahlawane et al., 2008). The WggR protein is indispensable for induction of expression of EPS II synthesis genes by both PhoB under P_i limitation and ExpR when the MucR regulator is present. However, WggR is not required for ExpR functioning in the absence of MucR. At a low population density, MucR inhibits EPS II synthesis until the ExpR/Sin system abolishes this effect (Mueller and González, 2011). A very close location of the binding sites for the WggR, ExpR, PhoB, and MucR regulators in the promoters of the galactoglucan synthesis genes enables competition for these target sites and interactions between these proteins. The complex regulation of expression of these genes allows modulating the synthesis of this polysaccharide in response to changing environmental conditions (Bahlawane et al., 2008; McIntosh et al., 2008).

Recently, it has been confirmed that the ExpR/Sin system also affects EPS I synthesis (Glenn et al., 2007). The ExpR protein positively regulates the expression of some *exo/exs* genes (*exoI* and *exsH*). Sequences resembling binding sites for ExpR have been identified in promoters of these genes (McIntosh et al., 2008; Gurich and González, 2009).

36.3.2 Regulation of EPS Synthesis in *R. leguminosarum*

In contrast to *S. meliloti*, data concerning regulation of EPS synthesis in *R. leguminosarum* are scarce. So far, only a few genes involved in this process have been identified; these include *rosR*, *exoR*, *expR*, and *pssB* genes located on the chromosome, and *psiA* and *psrA* genes located on the symbiotic megaplasmid (pSym) (Fig. 36.4) (Borthakur and Johnston, 1987; Reeve et al., 1997; Janczarek et al., 1999, Janczarek and Skorupska, 2007; Edwards et al., 2009).

Among these genes, *rosR* plays the most significant role in this process (Janczarek and Skorupska, 2007). This gene is located at a long distance from both the Pss-I cluster and *pssA* gene and constitutes an individual open-reading frame. *rosR* is a conserved gene present in strains belonging to all *R. leguminosarum* biovars (Janczarek et al., 2009b). It shares high similarity with *rosR* of *R. etli* (Bittinger et al., 1997), *rosR* of *R. gallicum* (Janczarek et al., 2009b), *rosAR* of *A. radiobacter* (Hussain and Johnston, 1997), and *mucR* of *S. meliloti* (Keller et al., 1995). A mutation in *rosR* of *R. leguminosarum* bv. *trifolii* causes several phenotypic defects, indicating an essential role of this gene in EPS synthesis and symbiosis. The lack of a functional *rosR* gene leads to a threefold decrease in EPS production, changes in membrane and extracellular protein profiles, and higher sensitivity to some osmolytes and surface-active detergents (Janczarek et al., 2010). In addition, the *rosR* mutant displays essentially decreased motility, colonization of host plant roots, and symbiotic defects. On the other hand, multiple *rosR* copies significantly enhance EPS production, competitiveness, and efficiency of *R. leguminosarum* bv. *trifolii* strains in symbiosis with clover (Janczarek et al., 2009a).

The expression of *rosR* is a complex process regulated by some environmental factors. A strong distal promoter and two additional regulatory elements (an upstream promoter sequence and a TGN extended −10 motif) are responsible for the high level of transcription of this gene (Janczarek and Skorupska, 2009). In addition, several other regulatory motifs were identified in the *rosR* upstream region, among them a RosR-box, a LysR motif, PHO-boxes, and motifs resembling the *E. coli* cAMP-CRP-binding site (Janczarek and Skorupska, 2007; 2011). RosR is a 15.7 kDa protein having a C_2H_2-type zinc-finger motif, which binds to a 22-bp palindromic sequence, called the RosR-box, in the *rosR* promoter region and represses the transcription of its own gene (Janczarek and Skorupska, 2007). Moreover, the carbon source, phosphate, and plant flavonoids affect *rosR* expression. The transcription of this gene increases in the presence of clover root exudates and NodD activator as well as PhoB activator (Janczarek and Skorupska, 2011). In addition, *rosR* expression is significantly decreased in the presence of glucose, indicating that this gene is regulated by catabolic repression (Janczarek and Skorupska, 2009). In *R. leguminosarum*, EPS production is also affected by the type of the carbon source, phosphate, and plant flavonoids, suggesting that the level of EPS synthesis in the presence of these environmental factors is a result of the RosR action (Janczarek and Skorupska, 2004; 2009). Positive regulation of EPS synthesis by the RosR protein is most probably an effect of simultaneous activation of *pssA* transcription (Janczarek and Skorupska, 2007) and inhibition of expression of the *pssB* and *exoR* genes involved in negative regulation of this process (Fig. 36.4) (Janczarek M., unpublished data). In addition, phosphate, ammonium, and clover root exudates slightly affect the expression of *pssB*, *pssP*, *pssO*, and *pssA* genes (Janczarek and Skorupska, 2004; Wielbo et al., 2004).

A genome-wide genetic screening performed in *R. etli* has indicated that RosR is essential in regulation of transcription of many diverse genes, including those required for the synthesis and modification of surface polysaccharides (*pssK*, *exoB*, *prsD*, and *plyA*) (Bittinger and Handelsman, 2000).

In *R. leguminosarum*, EPS synthesis is also regulated by *exoR*, which shows significant similarity to the *S. meliloti exoR* (Fig. 36.4). The ExoR protein most probably functions as a negative regulator of this process, since the *exoR* mutant of *R. leguminosarum* bv. *viciae* produces significantly more EPS than the wild-type strain under nitrogen starvation (Reeve et al., 1997).

pssB also seems to affect EPS production, although the precise role of this gene in this process has not been established. The *pssB* mutant of *R. leguminosarum* bv. *trifolii* produces slightly more EPS than the wild-type strain and elicits ineffective nodules on clover (Janczarek and Skorupska, 2001). This gene encodes a protein belonging to the family of inositol monophosphate phosphatases, which are responsible for conversion of inositol monophosphate to inositol. Catabolism of this compound is very important for competition and survival of rhizobial strains in soil.

Two other genes, *psiA* and *psrA*, have been found exclusively on the pSym plasmids of strains belonging to *R. leguminosarum* bv. *phaseoli*, indicating that this regulatory mechanism is specific only for this biovar (Borthakur and Johnston, 1987; Mimmack et al., 1994). A mutation in *psiA* does not affect EPS synthesis, but additional copies of this gene result in inhibition of EPS production and abolish nodulation of *Phaseolus* plants (Latchford et al., 1991). The negative effect of multiple *psiA* copies is overcome in the presence of extra copies of the *psrA* or *pssA* genes, indicating that a balanced number of copies of these genes is

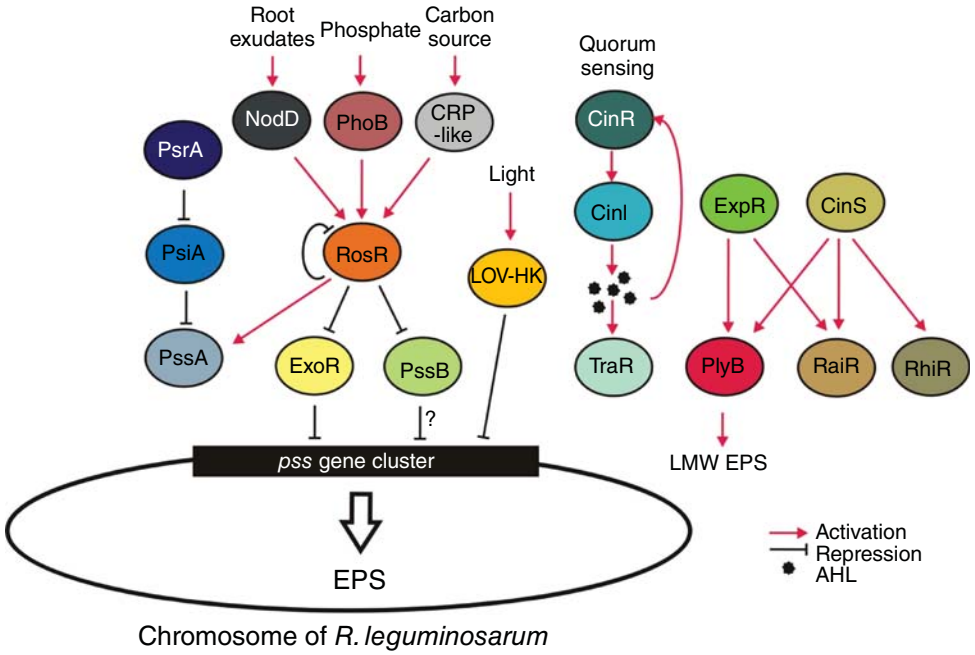

Figure 36.4 Regulation of EPS synthesis in *R. leguminosarum* (the figure is a modified version of the figure from the reference (Janczarek, 2011)).

required for a proper level of EPS synthesis. *psiA* codes for a small regulatory protein attached to the IM, which shows significant similarity to the *S. meliloti* ExoX. The same subcellular localization of PsiA as PssA suggests that this regulator most probably binds to this enzyme and inhibits its activity (Latchford et al., 1991). The transcription of *psiA* is repressed by the PsrA protein, which belongs to the family of transcriptional regulators containing a helix-turn-helix motif (Borthakur and Johnston, 1987; Mimmack et al., 1994). A *psrA* mutant produces less EPS than the wild type, whereas a strain carrying additional copies of this gene elicits nonnitrogen-fixing nodules on bean.

***36.3.2.1 Role of Quorum Sensing in the Synthesis of EPS in* R. leguminosarum.** In *R. leguminosarum* bv. *viciae*, four AHL-based quorum sensing systems have been identified (*cin*, *rhi*, *rai*, and *tra*) and shown to be involved in several cellular processes, among them are EPS production, plasmid transfer, and nodulation (Fig. 36.4) (Wisniewski-Dyé and Downie, 2002; see also Chapter 37). The *cin* system consisting of the CinR, CinI, and CinS proteins is at the top of this regulatory network and activates gene expression of the remaining quorum sensing systems. CinI is indispensable for AHL synthesis (*N*-(3-hydroxy-7-*cis*-tetradecenoyl)-L-homoserine lactone), whereas CinR induces *cinI* transcription in response to this signal. CinS is a small protein of an antirepressor function activating the transcription of regulatory genes *raiR* and *rhiR* of the *rai* and *rhi* systems and, additionally, the expression of *plyB* encoding an extracellular glycanase (Edwards et al., 2009; Frederix et al., 2011). This protein acts as an attenuator of the activity of the negative regulator PraR. Multiple *cinS* copies cause loss of colony mucoidy with colony aging, which is an effect of accumulation of the EPS-degrading PlyB enzyme.

Another regulatory protein ExpR also stimulates *raiR* and *plyB* expression, but this regulation does not require AHL (Edwards et al., 2009; Frederix et al., 2011). The ExpR protein of *R. leguminosarum* displays significant similarity to the *S. meliloti* ExpR. Nevertheless, several functional differences between these regulators have been observed. In contrast to the *S. meliloti expR* mutant, the *expR* mutant of *R. leguminosarum* forms colonies exhibiting morphology similar to that of the wild type. Moreover, the *R. leguminosarum* ExpR regulates a significantly lower number of genes than its homolog from *S. meliloti*, among them only *plyB* is associated with EPS production.

***36.3.2.2 The Influence of Light on EPS Synthesis in* R. leguminosarum.** Among the environmental factors, light is an essential signal that controls growth, development, and behavior of many different organisms (Purcell and Crosson, 2008). Light, oxygen, and voltage (LOV)-domain proteins are blue-light receptors found in higher plants, algae, fungi, and bacteria, which sense the wavelength and intensity of light and transduce this signal into various cellular pathways. Sequencing of bacterial genomes has shown that photoreceptor proteins are present not only in photosynthetic bacteria but also in heterotrophic bacteria such as *Brucella* and *Rhizobium*.

Recently, a *lov* gene encoding a sensor histidine kinase (LOV-HK) has been identified on the symbiotic megaplasmid pRL11 of *R. leguminosarum* bv. *viciae* 3841 (Bonomi et al., 2012). This protein contains the sensor LOV domain at the N-terminal end followed by an HK domain with histidine residue as a target site for autophosphorylation. Bonomi et al. (2012) have observed that light regulates EPS production, biofilm formation, flagella abundance, competitiveness, and nodulation in *R. leguminosarum* through this photoreceptor. Wild-type bacteria exposed to light show a substantial reduction in EPS production and biofilm formation, indicating that this signal negatively affects the synthesis of this polymer (Fig. 36.4). In contrast, the *lov* mutant shows no significant differences in the amounts of produced EPS and formed biofilm between light and dark conditions (Bonomi et al., 2012). But genes involved in EPS synthesis as targets for the LOV-HK protein have not been established so far. Light also affects competitiveness and nodulation in a *lov*-dependent manner, suggesting that this environmental signal is very important for adaptation of rhizobia to different ecological niches. However, genes encoding LOV-domain proteins are not common in rhizobial genomes, since only 5 among the 28 genomes sequenced to date harbor these genes (Krauss et al., 2009; Bonomi et al., 2012).

36.4 CONCLUSIONS

Exopolysaccharides secreted in large amounts to the environment by rhizobia are species-specific heteropolymers with several functions, including biofilm formation and protection against stress factors. Moreover, LMW EPS are biologically active forms essential for successful nodulation of leguminous plants (especially plants forming indeterminate-type nodules). So far, structures of EPS from several rhizobial species have been established. However, EPS synthesis and regulation of this process by environmental factors have been studied in detail only for one species – *S. meliloti*. These data indicate that EPS synthesis in this bacterium undergoes very complex regulation linked with other regulatory pathways such as quorum sensing and catabolite repression. In contrast, knowledge about proteins required for the synthesis of EPS as well as modulation of this process by different environmental factors in other rhizobial species is still scarce. Moreover, neither the precise function of LMW EPS nor plant receptors for this signal have been discovered so far.

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Chapter 37

Regulation of Symbiotically Important Functions by Quorum Sensing in the *Sinorhizobium meliloti*–Alfalfa Interaction

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37.1 INTRODUCTION

The rhizosphere of plants, rich in root exudates, attracts a variety of microorganisms that play a significant role in plant health.

A major component of root exudates are phenolic compounds called flavonoids (Cesco et al., 2010), which are important determinants of host specificity in the rhizobia-legume symbiosis (Long, 1996; see Chapter 50). Flavonoids act as bacterial chemoattractants and stimulate the expression of nodulation genes, which will set off the initial steps required for the establishment of the symbiosis. Upon recognition of a compatible host, the bacterial NodD regulator is able to sense the presence of flavonoids and activate the biosynthesis and secretion of Nod factors (Schlaman et al., 1992). These oligomers of 1,4-linked *N*-acetyl-glucosamine trigger a series of morphological changes in the plant root that leads to the formation of nodules, specialized organs where nitrogen fixation occurs (see Chapter 51). As rhizobia gather around the roots, the production of exopolysaccharides (see Chapter 36) allows bacteria to colonize the plant surfaces and invade root hairs through a tubular structure called the infection thread (Gage et al., 1996; Cheng and Walker, 1998; Gage, 2002; Gage, 2004; see Chapters 41, 57, 59). When infection threads reach the meristem, bacteria are released into the cell cytoplasm surrounded by a plant-derived membrane. Inside the nodules, bacteria differentiate into their endosymbiotic form, bacteroids (see Chapter 31), and begin to convert atmospheric nitrogen into reduced forms of this element that the plant can incorporate into organic compounds.

In addition to flavonoids and Nod factors, quorum sensing plays a role as part of the signaling process that allows the establishment of the symbiosis. Quorum sensing is defined as a population density mechanism that enables bacteria to coordinately regulate gene expression and modify behaviors. In response to flavonoids, the rhizobial population density increases in the rhizosphere, and it is at this junction that quorum sensing becomes an important player in the symbiotic process. In this chapter, we review how quorum sensing regulates symbiotically relevant functions in the *S. meliloti*-alfalfa interaction.

37.2 QUORUM SENSING IN GRAM-NEGATIVE BACTERIA

The production of signaling molecules allows bacteria to assess the size of their population and to respond to these changes as a group instead of as individuals. When the accumulation of these diffusible molecules or autoinducers reaches a threshold level, they activate a transcriptional regulator that in turn modifies the expression of target genes.

Biological Nitrogen Fixation, Volume 1, First Edition. Edited by Frans J. de Bruijn.

The best characterized quorum-sensing mechanism in Gram-negative bacteria involves the production of *N*-acyl homoserine lactones (AHLs). These autoinducers are synthesized by AHL synthases belonging to the LuxI family from *S*-adenosyl methionine (SAM) and an acylated acyl carrier (Hanzelka and Greenberg, 1996; More et al., 1996; Val and Cronan, 1998; Parsek et al., 1999; Fuqua et al., 2001; Withers et al., 2001). All AHL molecules described so far share a homoserine lactone head group attached to a fatty acyl chain. The length of the acyl chain (which varies from 4 to 18 carbons), the presence of substitutions in the third-carbon position (which can be a hydrogen, hydroxyl, or oxo group), and the backbone saturation confer specificity to the quorum-sensing signals (Nealson et al., 1970; Pearson et al., 1995; Schripsema et al., 1996; Puskas et al., 1997; Marketon et al., 2002; Thiel et al., 2009). When a threshold of AHLs has accumulated, they bind to a receptor protein of the LuxR family (Fuqua et al., 1996). LuxR-type receptors are transcriptional regulators that have an amino-terminal AHL-binding domain and a carboxyl-terminal DNA-binding motif (Shadel et al., 1990; Slock et al., 1990; Choi and Greenberg, 1991; Hanzelka and Greenberg, 1995). The AHL-receptor complex binds to DNA promoter sequences called *lux* boxes (inverted repeated sequences located about −40 nucleotides from the transcriptional start site), and by doing so activates or represses the expression of quorum-sensing-regulated genes (Devine et al., 1989; Fuqua et al., 1994; Stevens et al., 1994; Stevens and Greenberg, 1997; Egland and Greenberg, 1999; Luo and Farrand, 1999; Stevens et al., 1999; Egland and Greenberg, 2000; Pompeani et al., 2008).

In rhizobia, quorum sensing has been shown to control exopolysaccharide production (Marketon et al., 2002; Pellock et al., 2002; Marketon et al., 2003; Teplitski et al., 2003; Gao et al., 2005; Glenn et al., 2007; Bahlawane et al., 2008b; McIntosh et al., 2008; see Chapter 36), biofilm formation (Wang et al., 2004; Edwards et al., 2009; Rinaudi and González, 2009), motility (Sourjik et al., 2000; Hoang et al., 2004; Daniels et al., 2006; Hoang et al., 2008; Gurich and González, 2009; Nogales et al., 2012; see Chapter 33), plasmid transfer (Marketon and González, 2002; Wilkinson et al., 2002; Danino et al., 2003; He et al., 2003; Tun-Garrido et al., 2003), nodulation and nitrogen fixation (Cubo et al., 1992; Rosemeyer et al., 1998; Rodelas et al., 1999; Daniels et al., 2002; Loh et al., 2002; Zheng et al., 2006), among other functions.

37.3 THE ExpR/Sin QUORUM-SENSING SYSTEM IN *Sinorhizobium meliloti*

The most extensively studied quorum-sensing system in *S. meliloti* is the ExpR/Sin system. The *sinI*/*sinR* locus was originally identified in the commonly used Rm1021 strain (Marketon and González, 2002). It is composed of the transcriptional regulator SinR and the autoinducer synthase SinI, responsible for the synthesis of several long-chain AHLs (C12-HL, oxo-C14-HL, C16:1-HL, oxo-C16:1-HL, and C18-HL) (Marketon et al., 2002). Plant inoculation assays showed that disruption of *sinI* or *sinR* leads to a decrease in the total number of nitrogen-fixing nodules in alfalfa, as well as a delay in the appearance of such nodules, suggesting that quorum sensing plays a role in the establishment of a successful symbiosis (Marketon et al., 2002).

Numerous cellular processes require the activation of another LuxR homolog, ExpR, by the *sin*-encoded AHLs in *S. meliloti*. Among the genes regulated by the ExpR/Sin quorum-sensing system are those involved in exopolysaccharide production (see Chapter 36), motility, chemotaxis (see Chapter 33), nitrogen fixation, metabolism, and metal transport (Pellock et al., 2002; Marketon et al., 2003; Teplitski et al., 2003; Hoang et al., 2004; Gao et al., 2005; Glenn et al., 2007; Hoang et al., 2008; Gurich and González, 2009; Mueller and González, 2011). Curiously, unlike most wild-type strains tested, the widely studied Rm1021 strain carries an insertional element (ISRm2011-1) within the coding region of *expR*, which makes it "insensitive" to AHLs because it lacks a complete quorum-sensing system (Pellock et al., 2002; Marketon et al., 2003).

37.3.1 Regulation of Exopolysaccharide Production by the ExpR/Sin Quorum-Sensing System

S. meliloti is capable of producing two exopolysaccharides involved in the plant nodule invasion process, succinoglycan, and EPS II (see Chapter 36).

Succinoglycan is a polymer of octasaccharide repeating subunits. Each subunit is composed of seven glucose molecules and one galactose residue joined by β-1,4, β-1,3, and β-1,6 glycosidic linkages and decorated by acetyl, succinyl, and pyruvyl groups (Aman et al., 1981; Reuber and Walker, 1993b; Reinhold et al., 1994). All the genes required for succinoglycan biosynthesis (*exo* genes) have been identified and are located on the pSymB megaplasmid of *S. meliloti* (Leigh et al., 1987; Glucksmann et al., 1993; Reuber and Walker, 1993a,1993b; see Chapter 36). This exopolysaccharide is produced in two distinct fractions: one of high molecular weight (HMW) and the other of low molecular weight (LMW). The symbiotically active form necessary for the establishment of the symbiosis with alfalfa is the LMW fraction of succinoglycan and consists of trimers of the octasaccharide (Battisti et al., 1992; González et al., 1998; Wang et al., 1999; see Chapter 36). Rm1021 *exo* mutants are symbiotically deficient, eliciting the formation of small nodules devoid of bacteria. Studies using

GFP-labeled *S. meliloti* revealed that strains impaired in the production of succinoglycan fail to initiate and elongate infection threads during the invasion of alfalfa roots (Leigh et al., 1985; Yang et al., 1992; Cheng and Walker, 1998; Pellock et al., 2000). A whole-genome expression profile of *S. meliloti* showed that the ExpR/Sin quorum-sensing system regulates genes involved in the production of succinoglycan and its LMW fraction (Hoang et al., 2004; Gurich and González, 2009). These results were confirmed by Glenn and coworkers, who observed a fivefold reduction in the production of succinoglycan in a strain deficient in the synthesis of AHLs. Interestingly, the expression of *exsH* and *exoK*, the endo-1,3-1,4-β-glycanases, necessary for the production of the symbiotically active LMW fraction of succinoglycan was also induced by the ExpR/Sin system (Glenn et al., 2007; Gurich and González, 2009). Disruption of *expR* and *sinI* also caused a delay in the appearance of the fluorescent halo surrounding succinoglycan-producing colonies, indicative of a reduction in the synthesis of the LMW fraction of the exopolysaccharide (Glenn et al., 2007).

In the absence of succinoglycan, EPS II can mediate the establishment of the symbiosis between *S. meliloti* and alfalfa (Zhan et al., 1989; see Chapter 36). EPS II is an exopolysaccharide of repeating dimer subunits made of an acetylated glucose and a pyruvylated galactose residue connected by α-1,3 and β-1,3 glycosidic bonds (Glazebrook and Walker, 1989; Her et al., 1990). Biosynthesis of EPS II is directed by the *exp* operon, also located on the pSymB plasmid (Glazebrook and Walker, 1989). As with succinoglycan, EPS II is produced in HMW and LMW forms. The LMW fraction, consisting of 15–20 disaccharide subunits of EPS II, is able to rescue nodule invasion in an exopolysaccharide-deficient strain (González et al., 1996). Production of EPS II and its symbiotically active LMW fraction requires the presence of an intact ExpR/Sin quorum-sensing system (Pellock et al., 2002; Marketon et al., 2003; see Chapter 36). Synthesis of EPS II is abolished in a *sinI* mutant, but this deficiency can be complemented by the addition of AHLs extracted from a *sinI*-proficient strain or by synthetic C16:1-HSL (Marketon et al., 2003). The mechanism by which the ExpR/Sin system induces the production of EPS II and the active LMW form of the exopolysaccharide was recently revealed by Mueller and González. The *exp* gene cluster is composed of the operons *expE*, *expA*, and *expD*, which are required for the structural biosynthesis of EPS II, as well as for the *expG-expC* operon, which encodes a transcriptional regulator and a glycosyl transferase, respectively (Glazebrook and Walker, 1989; Becker et al., 1997; Rüberg et al., 1999; Bahlawane et al., 2008a). In a quorum-sensing-proficient strain, the regulator MucR represses the synthesis of EPS II at low-population densities through direct interactions with the promoter regions of *expE*, *expA*, and *expD* (Rüberg et al., 1999; Bahlawane et al., 2008a). When quorum is reached, the ExpR-AHL complex activates the expression of the transcriptional regulator encoded by *expG*, which removes, at the transcriptional level, the MucR repression of EPS II production. Increased levels of expression of the *expC*-encoded glycosyl transferase cotranscribed with *expG*, along with maintained expression of the structural *exp* genes, results in the synthesis of the symbiotically active LMW form of EPS II (Mueller and González, 2011).

37.3.2 Regulation of Biofilm Formation by the ExpR/Sin Quorum-Sensing System

The ExpR/Sin system also controls biofilm formation in *S. meliloti*, mainly through the regulation of EPS II production (Rinaudi and González, 2009). Disruption of *sinI* or *expR* results in a dramatic reduction in biofilm formation. An identical phenotype was observed when EPS II synthesis was blocked by introducing a mutation in the *expA* gene. Moreover, only a quorum-sensing-proficient strain was able to develop highly structured and organized biofilms when evaluated by confocal laser scanning microscopy. Mutants unable to produce EPS II (*sinI*, *expR*, and *expA*) or the active LMW form of the exopolysaccharide (*mucR*) failed to aggregate and form biofilms. A reduction in the attachment to roots of the legume host was also correlated with the mutant's inability to produce EPS II. These results suggest that the quorum-sensing-regulated production of the symbiotically important form of EPS II not only mediates invasion of alfalfa but also contributes to the colonization of root surfaces (Rinaudi and González, 2009). Therefore, the ExpR/Sin quorum-sensing-mediated mechanism that regulates EPS II production in *S. meliloti* ensures that LMW EPS II is produced at the adequate population densities for root colonization and invasion of the host.

37.3.3 Regulation of Motility by the ExpR/Sin Quorum-Sensing System

The expression of many genes involved in the synthesis of flagella filaments (belonging to the *flb* and *fla* operons), the basal body and hook (*flgEKL* and *fliL* genes), as well as chemotaxis (*cheY1* gene) are dependent on the existence of a functional ExpR regulator, and downregulated in the presence of Sin-AHLs (Hoang et al., 2004; see Chapter 33). Previous studies have established that the LuxR-type transcriptional regulators VisN/R and the OmpR-like transcription factor Rem are activators of motility in *S. meliloti* (Sourjik et al., 2000; Rotter et al., 2006). The expression of *visN*, *visR*, and *rem* was downregulated, in a population density-dependent manner, in a *sinI* mutant compared to the quorum-sensing-proficient strain (Hoang et al., 2008). This suggests that when sufficient AHLs have accumulated, quorum sensing acts upstream of these

regulators and controls motility via the VisN/R-Rem regulatory pathway. Recent work showed that the ExpR-AHL complex regulates motility directly by binding to the promoter region of the VisN transcriptional regulator, resulting in the repression of flagella synthesis (Bahlawane et al., 2008b). It has been proposed that free ExpR and AHL-activated ExpR compete for control of motility genes (Gurich and González, 2009). At low-population densities, the basal levels of AHLs leave ExpR available to act as an activator of motility. As the concentration of AHLs increases, ExpR binds upstream of the *visN/R* operon and becomes a repressor, preventing the activation of motility and chemotaxis genes (see also Chapter 33).

A *sinI* mutant is highly motile and unable to repress the synthesis of flagella at high-population densities, which is detrimental to successful plant invasion (Hoang et al., 2008; Gurich and González, 2009). Blocking flagellar synthesis in a *sinI* strain (by disrupting the filament biosynthetic genes *flaA* and *flaB*) completely restored its competency for establishing symbiosis to wild-type levels. Blocking flagella rotation (not its synthesis) in the *sinI* mutant (*sinI motA*) was not able to restore the invasion efficiency in this strain (Gurich and González, 2009). These results taken together strongly suggest that the inability to shut down flagella in *S. meliloti* interferes with alfalfa plant invasion.

37.3.4 The ExpR/Sin Quorum-Sensing System Inside the Nodules

Genome-wide expression profile analyses revealed that the expression of all the ExpR/Sin quorum-sensing components (*sinR*, *sinI*, and *expR*), and quorum-sensing-regulated genes (such as those involved in EPS II synthesis) was strongly repressed in bacteroids and only detected in free-living cells (Gurich and González, 2009). The same studies also confirmed the activation of genes involved in nitrogen fixation and microoxic respiration in bacteroids compared to free-living cells, while metabolic, ribosomal, cell division, and translational genes were downregulated in the nodule (Gurich and González, 2009). Evidence suggests that the ExpR/Sin system plays a major role regulating the expression of genes necessary for bacterial survival in the soil, colonization of the host, and invasion of alfalfa roots, but it is inactivated once rhizobia enters the plant and the symbiosis has been established.

37.4 THE TRA QUORUM-SENSING SYSTEM IN *Sinorhizobium meliloti*

A second quorum-sensing system has been identified in *S. meliloti*. The Tra system, named for its homology to the equally named quorum-sensing system in *Agrobacterium tumefaciens* and *Rhizobium* strain NGR234, is located on the self-transmissible, nonsymbiotic pRme41a plasmid carried by *S. meliloti* Rm41 derivatives (Marketon and González, 2002).

This system is composed of three regulatory genes: *traI*, *traR*, and *traM* (Fig. 37.1). At low-population densities, TraM binds to and inactivates TraR, preventing it from inducing the *trb* operon until a high population is reached. At high-population densities, *traI*-encoded AHLs (3-oxo-C8-HSL, 3-OH-C8-HSL, and C8-HSL) accumulate to a level where they effectively bind to and activate the response regulator TraR. The TraR-AHL complex then upregulates the expression of the *trb* operon, which contains genes that encode for the formation of the mating pore and the conjugal tube, structural components necessary for the transfer of the plasmid (Marketon and González, 2002).

37.5 OTHER LuxR HOMOLOGS IN *S. meliloti*

The sequenced genome of *S. meliloti* Rm1021 reveals the presence of four additional LuxR homologs. As with the

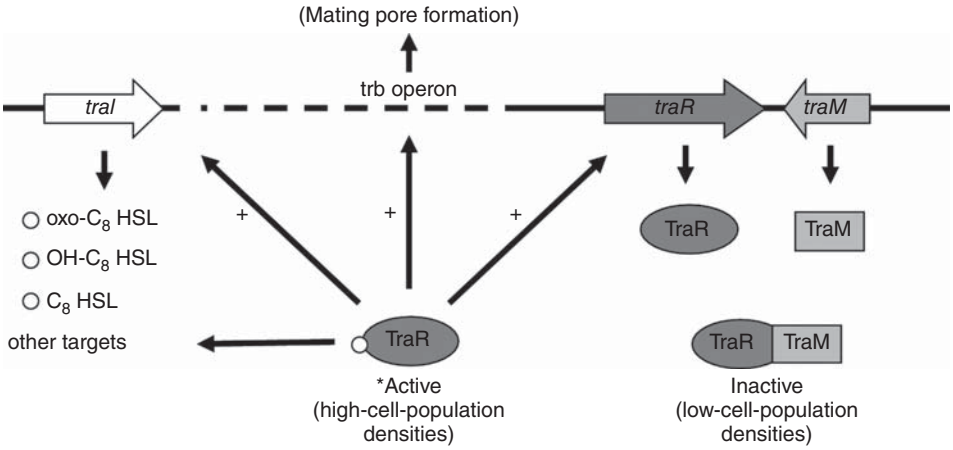

Figure 37.1 Tra quorum-sensing system in *S. meliloti*. This system controls conjugal plasmid transfer through the action of the *tra* genes, located in the pRme41a plasmid. TraI produces short-chain AHLs, and it is regulated by TraR. TraM is a repressor of TraR at low-population densities, which ensures that the Tra system is active only at the high-population densities, necessary for the conjugal transfer of pRme41a.

other two characterized LuxR-type regulators, SinR and ExpR, these LuxR homologs are located in the chromosome and are encoded by the SMc04032, SMc00878, SMc00877, and SMc00658 genes. However, none of them are directly associated with a known AHL synthase in *S. meliloti*, and therefore, they are considered orphan LuxR regulators (Galibert et al., 2001; Fuqua, 2006; Patankar and González, 2009b; Subramoni and Venturi, 2009).

Recent work has proposed a role for one of these orphan regulators (SMc04032), named NesR (Patankar and González, 2009a). Genome-wide expression analysis showed that NesR is involved in the active methyl cycle within the methionine biosynthetic pathway, responsible for the production of the important AHL precursor SAM. In the absence of an intact *nesR*, four genes were downregulated in the mutant when compared to the wild type. These encode for a methionine synthase (*metH*), a betaine methyltransferase (*bmt*), an *S*-adenosyl-L-methionine synthase (*metK*), and a methylene tetrahydrofolate reductase (*metF*). NesR does not seem to be associated with the production or regulation of the Sin-AHLs. The introduction of a mutation in the *nesR* gene does not cause a growth defect in the mutant; however, the absence of a functional copy of the regulator renders the strain less capable of tolerating hyperosmotic and detergent stresses. Although plant nodulation assays showed that the *nesR* mutant is as efficient as the wild type at establishing a successful symbiosis with alfalfa, co-inoculation assays revealed that the mutant was less competitive for nodulation than the wild-type strain of *S. meliloti*. Taken together, these results suggest NesR is a modulator of metabolic fitness, and it plays a role in *S. meliloti*'s stress adaptation and competition for plant nodulation (Patankar and González, 2009a).

Preliminary results indicate that SMc00878 controls the transcription of genes from the denitrification pathway in *S. meliloti* (A. Patankar and J.E. González, unpublished results), while the roles of SMc00877 and SMc00658 remain to be elucidated.

37.6 CONCLUSION

Plants live in close association with numerous microorganisms that inhabit the soil. Complex signaling mechanisms allow them to recruit beneficial microbes, which improve nutrient uptake, prevent colonization by pathogens, and modulate host defense responses (Berendsen et al., 2012). Among these beneficial microbes are rhizobia, nitrogen-fixing soil bacteria, able to establish a symbiosis with legume plants. Important signaling events are triggered in the rhizosphere by plant flavonoids, as well as bacterial Nod factors, exopolysaccharides, and quorum-sensing molecules.

The ExpR/Sin quorum-sensing system has been shown to regulate numerous symbiotically important functions in free-living *S. meliloti*. Such functions include the production of exopolysaccharides, biofilm formation, motility, and chemotaxis (Fig. 37.2). By being able to use AHLs to monitor the size of its population, *S. meliloti* allows the synthesis of flagella and the activation of motility at low-population densities, when cells are exploring new niches, searching for nutrients or trying to reach their host. As the population increases around the legume roots, motility is downregulated while the synthesis of the active LMW fractions of succinoglycan and EPS II is induced, events necessary for efficient plant invasion. Once invasion has occurred and the symbiosis has been established, all metabolic functions of

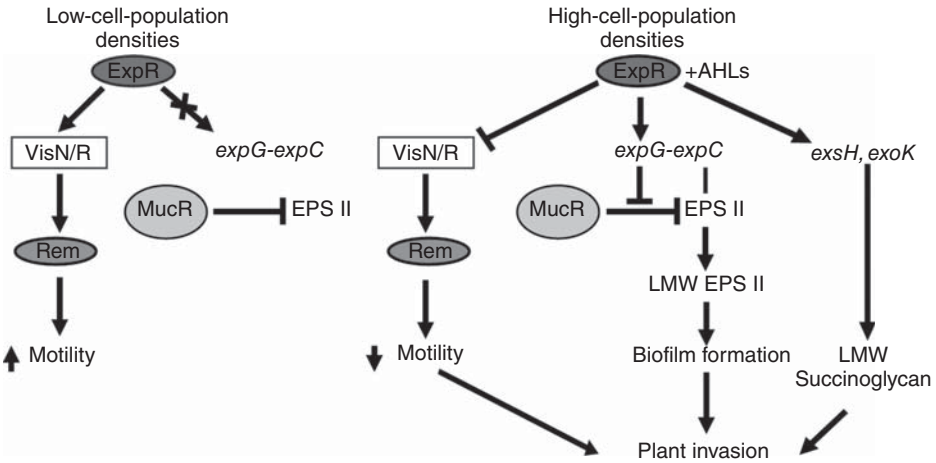

Figure 37.2 Quorum-sensing regulation of exopolysaccharide production, biofilm formation, and motility in *S. meliloti*. Low concentrations of AHLs, insufficient to activate ExpR, allow bacteria to be motile, a requirement for movement toward the legume host. At high-population densities, the ExpR-AHL complex blocks expression of *visN/R* while derepressing EPS II synthesis from MucR, leading to the production of the symbiotically active exopolysaccharides. Lack of flagella production and the presence of LMW succinoglycan and/or EPS II are necessary for the establishment of a successful symbiosis between *S. meliloti* and alfalfa plants.

the bacteroids are directed toward nitrogen fixation, while quorum sensing and quorum-sensing-regulated genes are repressed inside the nodule.

Little is known about the nonsymbiotic pRme41a plasmid present in *S. meliloti* Rm41, which carries the Tra quorum-sensing system (Marketon and González, 2002). *traI*-encoded AHLs, in conjunction with the regulator TraR, have been linked to the conjugal transfer of pRme41a (Marketon and González, 2002). It has been proposed that this plasmid also carries calystegin catabolic genes, a secondary metabolite found in the root exudates of some plants, and that the ability to utilize less-conventional substrates by strains such as Rm41 could provide them a selective advantage under natural conditions (Tepfer et al., 1988). The now available sequence of Rm41 and pRme41a could shed light on other potentially important functions harbored by this cryptic plasmid.

Finally, the genome of *S. meliloti* shows the presence of four orphan LuxR homologs: SMc04032 (NesR), SMc00658, SMc00877, and SMc00878 (Galibert et al., 2001). NesR was shown to affect stress adaptation and competition for nodulation (Patankar and González, 2009a), while the processes regulated by SMc00878 mostly fall within the denitrification pathway of *S. meliloti* (A. Patankar and J.E. González, unpublished results). The roles for SMc00658 and SMc00877, as well as the signal(s) responsible for activation of these orphan LuxR homologs remain to be identified.

The natural aggregation of *S. meliloti* that occurs in the plant host root milieu is the trigger that activates the bacterium's quorum-sensing system to coordinate a series of phenotypes essential for the successful invasion of alfalfa nodules. Once *S. meliloti* enters the nodule and directs its resources to the nitrogen-fixing process, quorum sensing and the genes regulated via its signaling system are no longer necessary, and they are inactivated by a mechanism still to be determined.

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Chapter 38

Lumichrome: A Bacterial Signal Molecule Influencing Plant Growth

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38.1 INTRODUCTION

Both plants and bacteria use various mechanisms to overcome environmental limitations such as low nutrients. For example, plants secrete a mixture of molecules that promote nutrient mobilization for uptake by roots (Marschner, 1995; Dakora and Phillips, 2002). Many diazotrophs, including rhizobia, have been shown to use chemical molecules in their exudates to affect changes in plant development. For example, many bacterial species secrete specialized compounds such as siderophores to enhance Fe acquisition (Jurkevitch et al., 1986). Rhizobial bacteria are also known to release growth-promoting molecules (Fig. 38.1) such as gibberellins and cytokinins (Phillips and Torrey, 1970; 1972; Dart, 1974; Lynch and Clark, 1984) that promote bacterial cell growth and massively proliferate plant root hair production (Yanni et al., 2001) for increased water and nutrient uptake through an enhanced root absorptive capacity. In this study, the role of lumichrome as a signal molecule in plant development is assessed together with riboflavin and indole acetic acid (Fig. 38.1), two other bacterial metabolites that are important in rhizosphere functioning of symbiotic legumes.

38.2 COMPONENTS OF RHIZOBIAL EXUDATES AND THEIR EFFECTS ON PLANTS

In addition to bacterial hormones, a number of biologically active novel molecules have been purified and identified from rhizobial exudates that stimulate cell growth and nodule organogenesis (De Jong et al., 1993; Daychok et al., 2000). For example, lipochito-oligosaccharide molecules isolated from rhizobia (Fig. 38.1) have been reported to stimulate seed germination (Zhang and Smith, 2002) and seedling development in both monocots and dicots (Smith et al., 2002; see Chapter 107). In addition to *nod* gene induction, purified Nod factors have been found to morphogenically elicit nodule formation on host plants in the absence of rhizobial cells (Denarie et al., 1996). Applying 10^{-7} M or 10^{-9} M concentration of Nod factors to soybean plants increased root mass by 7–16% and root length by 34–44% (Smith et al., 2002; see Chapter 107). Similarly, spraying leaves of soybean, common bean, maize, rice, canola, apple, and grape plants with submicromolar concentrations [10^{-6}, 10^{-8}, or 10^{-10} M] of Nod factors increased photosynthetic rates by 10–20% (Smith et al., 2002) and resulted in a 40% increase in grain yield of a field-grown soybean crop (Smith et al., 2002; see Chapter 107). Furthermore, Nod factors have been found to induce the expression of genes involved in the phenylpropanoid pathway (Savoure et al., 1994; Spaink and Lugtenberg, 1994), with potential for increasing phytoalexin production, and hence greater protection of plants against pathogens (Dakora and Phillips, 1996). It has also been shown that, even at low concentrations [10^{-7} nM], Nod factors can promote AM colonization of both nodulating and nonnodulating plants (Xie et al., 1995), suggesting a role for this rhizobial metabolite in the establishment of mycorrhizal symbiosis (Parniske, 2008; see Chapter 108).

In addition to Nod factors, other metabolites have been identified in rhizobial exudates, and these include vitamins and amino acids. Although the presence of these compounds

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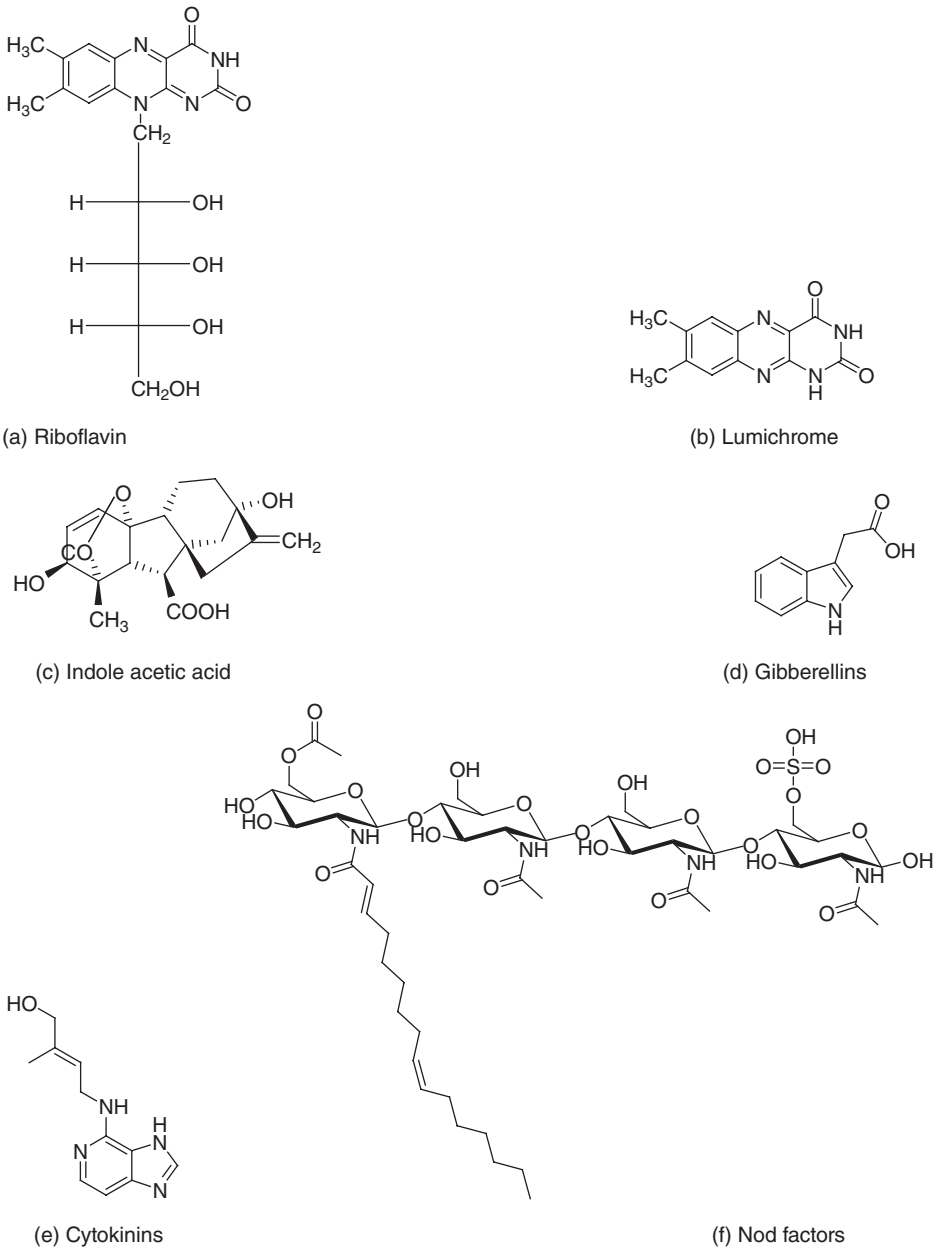

Figure 38.1 Structures of rhizobial molecules affecting plant function.

in bacterial cultures was reported a long time ago, it is only recently that their functional role in plant growth and metabolism has been documented. For example, the observed synthesis of the vitamins riboflavin and thiamine by root-nodule bacteria for cell growth occurred about 75 years ago (West and Wilson, 1938). Five years after that, riboflavin was isolated from field soil and was shown to be absorbed by plant roots and translocated to shoots (Carpenter, 1943). It was not until 35 years later that riboflavin was reported to stimulate plant growth (Rao, 1973). Since then, different vitamins such as thiamine, riboflavin, niacin, biotin, and pantothenic acid, as well as amino acids such as glutamate, lysine, arginine, tryptophan, and methionine have been isolated from culture filtrate of several bacteria species, including *Sinorhizobium meliloti*, *Rhizobium leguminosarum* bv. *viceae*, *Azospirillum brasilense*, *Azotobacter vinelandii*, and *Pseudomonas fluorescens*, (Rodelas et al., 1993; Sierra et al., 1999; Yang et al., 2002). Three other biologically active molecules that have been identified from rhizobial exudates and found to promote plant growth include lumichrome, riboflavin, and indole acetic acid (Phillips et al., 1999; Matiru and Dakora, 2005a,b; Kanu et al., 2007; Kanu and Dakora, 2009; see Fig. 38.1).

38.3 LUMICHROME: A RHIZOBIAL SIGNAL MOLECULE INVOLVED IN PLANT DEVELOPMENT

Lumichrome is a molecule commonly synthesized by both microbes and plants, and is also a known degradation product of the vitamin riboflavin (Phillips et al., 1999) (see Fig. 38.1). Because riboflavin is easily converted enzymatically or photochemically into lumichrome (Yagi, 1962; Yanagita and Foster, 1956), any discussion of lumichrome must include riboflavin. Applying purified lumichrome from *Sinorhizobium meliloti* exudate to roots of alfalfa seedlings increases root respiration by 11–30% and promotes plant growth by 8–18% (Phillips et al., 1999). The enhanced plant growth has been attributed to an increased net C assimilation, possibly via PEP carboxylase activity (Phillips et al., 1999).

Plants seem to show a mixed response to lumichrome application. While this molecule significantly increases root respiration in maize plants (Phillips et al., 1999; Matiru and Dakora, 2005a), it decreases root respiration in lupin and has no affect on cowpea, soybean, Bambara groundnut, pea, and sorghum (Matiru and Dakora, 2005a). Inoculating the roots of these monocots and dicots with infective rhizobial cells produces the same results as obtained with lumichrome application, in that maize shows significantly increased root respiration; lupin a decreased rate; and cowpea, soybean, Bambara groundnut, pea, and sorghum remain unaffected in their root respiration (Matiru and Dakora, 2005a). This clearly indicates that the observed changes in root respiration with bacterial inoculation are caused by the lumichrome molecule released by symbiotic rhizobia. Furthermore, both lumichrome and riboflavin have been implicated as quorum-sensing molecules in bacteria (Rajamani et al., 2008). The independent role of riboflavin as a signal molecule has also been underscored by the finding that *Sinorhizobium meliloti* strains carrying extra copies of the riboflavin biosynthesis gene *ribBA* release 15% more riboflavin than the wild type and were 55% more efficient in alfalfa root colonization for nodule formation (Yang et al., 2002).

Treating the roots of cowpea, Bambara groundnut, soybean, sorghum, and maize plants with 10 nM purified lumichrome and 10 ml of infective rhizobial cells [0.2 OD_{600}] for 44 h in growth chambers increases stomatal conductance and leaf transpiration rates in cowpea, but decreases both parameters in Bambara groundnut, soybean, and maize (Matiru and Dakora, 2005b). In the latter study, the effect of bacterial inoculation closely mirrored that of 10 nM lumichrome application, again indicating that rhizobial effects on these physiological changes (whether in nature or under experimental conditions) is most likely because of the lumichrome molecule released by symbiotic rhizobia in the rhizosphere. Thus, the finding that rhizobial inoculation in the field alleviates the effects of water stress in symbiotic legumes (Figueiredo et al., 1999) could be attributed to strain secretion of lumichrome that decreased stomatal conductance and reduced water loss.

The supply of 5 nM lumichrome to roots of cowpea and soybean seedlings also causes early initiation of trifoliate leaf development, expansion in unifoliate and trifoliate leaves, and increased stem elongation (Fig. 38.2), resulting in an increase in the total biomass of shoot and plant relative to control. Lumichrome [5 nM] also increases leaf area in maize and sorghum, and thus raises shoot and total biomass but has no effect on the leaf area of the other cereals. Root growth is also stimulated in sorghum and millet by the supply of 5 nM lumichrome. However, higher doses of lumichrome [50 nM] depresses development of unifoliate leaves in soybean, the second trifoliate leaf in cowpea (Fig. 38.2), and shoot biomass in soybean. The 50 nM concentration also consistently decreases root development in cowpea and millet, but has no effect on the other species. The data also show that the biological effect of lumichrome on plants is not age specific as growth of 11-day-old and 37-day-old sorghum; 23-day-old and 37-day-old soybean; 23-day-old and 37-day-old millet; and 11-day-old and 37-day-old cowpea are significantly increased by lumichrome supply at 5 nM concentration. Unlike the legumes, however, the supply of 5 nM lumichrome markedly increases [$P < 0.05$] root growth in cereals, especially sorghum and millet. These data further show that lumichrome is a rhizosphere signal molecule that affects seedling development in both monocots and dicots.

At the metabolic level, root application of lumichrome increases starch accumulation in roots of both *Lotus* and tomato, and the molecule has been suggested to be involved in carbon partitioning and the modulation of carbon fluxes in infected symbiotic plant cells (Gouws et al., 2012). This argument has been reinforced by the fact that *Lotus*-treated roots showed a reduction in organic acids and amino acids, all carbonaceous compounds. However, root treatment with lumichrome increases ethylene evolution rates in *Lotus*, but not in tomato (Gouws et al., 2012). Taken together, these findings show that bacteria are capable of producing various organic molecules that alter plant development. From the recent discoveries of active bacterial metabolites, it has become clear that apart from the classical phytohormones (e.g., auxins, cytokinins, gibberellins, and abscisic acid), additional signaling molecules exist that influence plant development. Although Phillips et al. (1999) attributed the enhanced plant growth from lumichrome application to an increased net C assimilation via PEP carboxylase activity, the dramatic expansion in unifoliate and trifoliate leaves and the increased stem elongation observed with lumichrome application to cowpea and soybean would seem to suggest that this molecule stimulates plant growth via cell division and cell expansion, as happens with classical phytohormones

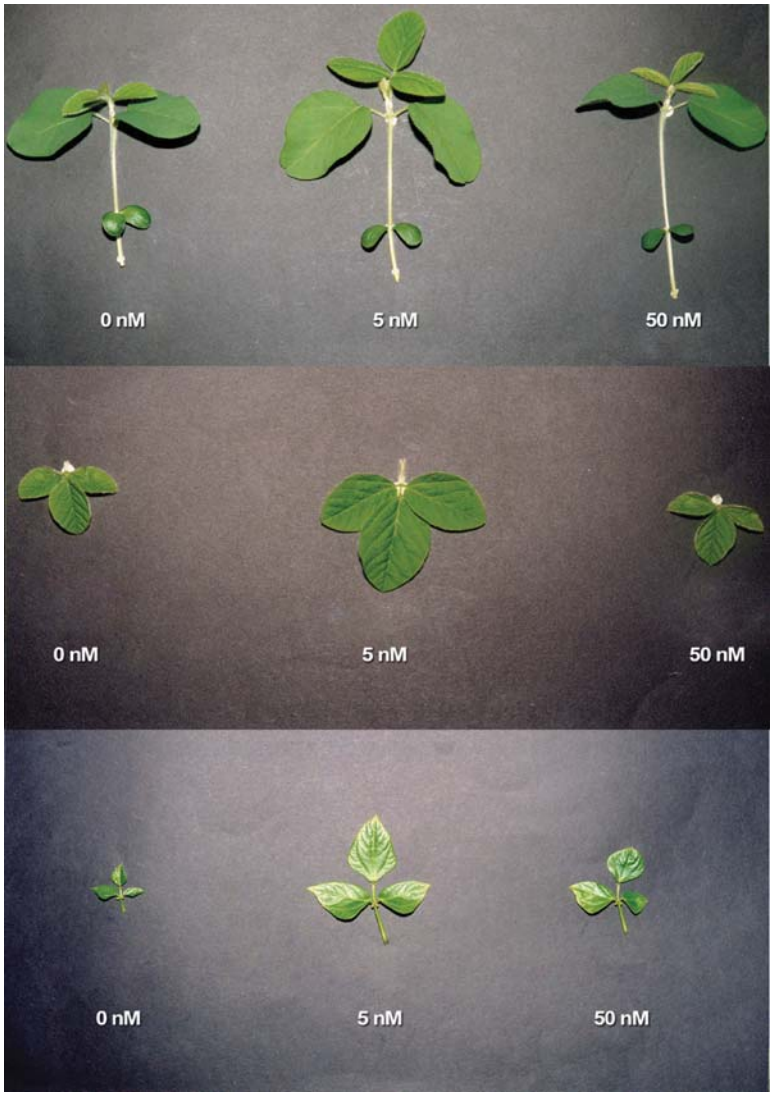

Figure 38.2 Lumichrome-induced changes in (a) stem elongation and trifoliate leaf development in soybean seedlings, (b) first trifoliate leaf initiation and expansion in soybean seedlings, and (c) first trifoliate leaf initiation and expansion in cowpea seedlings. (*Source:* The New Phytologist; see Matiru and Dakora, 2005b.)

(Letham et al., 1978; Ross et al., 2002; Campanoni et al., 2003; van der Graaff et al., 2003).

38.4 EFFECT OF LUMICHROME ON PLANT GROWTH AND PRODUCTIVITY

The increase in stem elongation, early initiation, and rapid expansion of trifoliate leaves observed with the application of 5 nM lumichrome to cowpea and soybean plants results in a twofold accumulation of dry matter in trifoliate leaves relative to the zero-lumichrome control (Matiru and Dakora, 2005b). In addition to legumes, lumichrome could also stimulate seedling development in cereal crops such as millet, sorghum, and maize. As a result, whole-plant dry matter yield of these cereal species receiving 5 nM lumichrome is greater compared to control (Matiru and Dakora, 2005b). At 5 nM lumichrome supply, root growth is much greater in the cereals (especially in millet and sorghum) than the legumes, suggesting that, in the former, lumichrome application alters assimilate partitioning in favor of root development. Gouws et al. (2012) also observed an increase in dry matter accumulation following lumichrome application to *Lotus* and tomato. The observed plant growth promotion by lumichrome in both monocots and dicots suggests that cereals could potentially benefit from lumichrome released by symbiotic rhizobia, in addition to N-fixed in cropping systems. Its growth-promoting effect on both monocots and dicots also suggests that lumichrome is capable of influencing plant rhizospheres in both natural and agricultural settings.

Foliar application of lumichrome at 10^{-6} M concentration significantly increases shoot and total dry matter yield of field-grown soybean plants (Khan et al., 2008). The increased accumulation of dry matter is partly due to a marked increase

in leaf area with lumichrome application (Khan et al., 2008). As found with cowpea and soybean (Matiru and Dakora, 2005a), the observed increase in plant growth and Fe uptake following sorghum inoculation with infective rhizobia (Matiru and Dakora, 2004) could be attributed to lumichrome secreted by the introduced bacterial cells.

38.5 EFFECT OF ENVIRONMENTAL FACTORS ON THE SYNTHESIS AND RELEASE OF LUMICHROME BY RHIZOBIA AND NODULE ENDOPHYTES

There are many factors affecting the production and release of metabolites by soil bacteria. For example, the synthesis and extracellular release of lumichrome, riboflavin, and indole acetic acid by rhizobia have been found to differ between and among species and strains (Kanu et al., 2007). In some studies, there is generally greater production of lumichrome, riboflavin, and indole acetic acid by N_2-fixing bacteria than those unable to nodulate *Psoralea pinnata* and sirato (Shokri and Emtiazi, 2010; Kanu and Dakora, 2012), a finding consistent with their role in symbiotic N_2 fixation (Phillips et al., 1999; Lambrecht et al., 2000; Matiru and Dakora, 2005a,b). For example, N_2-fixing strain TUT57pp produces 2.2-fold and 3.2-fold more indole acetic acid than the nonnodulating isolates TUT65prp and TUT33pap, respectively (Kanu and Dakora, 2012). Kanu and Dakora (2009) found that bacterial isolates from *Psoralea* nodules obtained from the Cape fynbos differ in their levels of secretion of lumichrome, riboflavin, and indole acetic acid, as well as in their exudation response to pH, salinity, and temperature. For example, while bacterial isolate AS2 from *Psoralea* nodules can produce greater amounts of lumichrome at both pH 5.1 and 8.1, strains RT1 and P1 secrete more lumichrome per cell at only pH 8.1. Strains AP1 and RP2 have also been found to produce more riboflavin at pH 8.1 than at pH 5.1; conversely strain RT1 produces greater amounts of riboflavin at pH 8.1 than at pH 5.1. Taken together, measured levels of lumichrome and riboflavin secreted by *Psoralea* bacterial isolates range from 0.1 to 15 nM (Kanu and Dakora, 2012).

Furthermore, two *P. repens* strains (RP1 and RP2] isolated from a very saline environment close to the Indian Ocean secrete large amounts of lumichrome and riboflavin at both low-salinity and high-salinity levels (Kanu and Dakora, 2009). Although the concentration of indole acetic acid produced by *Psoralea* isolates is greater at high acidity and high temperatures, with lumichrome the levels are much elevated at lower temperature (10 °C) than at high temperature (30 °C) (Kanu and Dakora, 2009). The greater lumichrome production at lower temperature is not surprising as Nod factors produced by *Bradyrhizobium aspalati* (now *Burkholderia tuberum*) isolated from *Aspalathus canosa* in the Cape fynbos have been found to be similar at 12 °C and 28 °C (Boone et al., 1999). This can be explained by the fact that legume nodulation in the Cape occurs with the winter rains when temperatures are around 10–15 °C. As found with salinity, plants and their associated microbial endophytes of the Cape fynbos seem metabolically adapted to the low winter temperatures.

Although we know the effect of N and P nutrition on Nod factor production in symbiotic rhizobia (McKay and Djordjevic, 1993; see Chapter 46), little information currently exists on the effects of these nutrients on the biosynthesis of other symbiotically important metabolites such as lumichrome, riboflavin, and indole acetic acid. A recent study has found marked variation in the secretion of lumichrome, riboflavin, and indole acetic acid by nodulating and nonnodulating bacteria isolated from *Psoralea* species (Shokri and Emtiazi, 2010; Kanu and Dakora, 2012). In another study (Kanu and Dakora, 2012), we have found much greater concentrations of lumichrome and riboflavin in exudates of five N_2-fixing and 11 nonnodulating bacterial strains grown at high P [5.7 mM] than at low P [1.4 mM]. The five N_2-fixing strains also differ in their levels of extracellular lumichrome secretion, with TUT23prt releasing the most lumichrome at both low P and high P, and TUT18pac the least.

Ammonium nutrition [28.1 mM or 112.0 mM NH_4^+] has no effect on the riboflavin synthesis and release (Kanu and Dakora, 2012), a finding consistent with the lack of response of Nod factor secretion to ammonium supply (McKay and Djordjevic, 1993).

However, lumichrome production is markedly affected by ammonium nutrition (Kanu and Dakora, 2012). Although some strains produce more or less lumichrome with ammonium supply, strains TUT23prt and TUT33pap can produce significant amount of lumichrome at both low and high ammonium concentrations (Kanu and Dakora, 2012). The level of lumichrome and riboflavin production by the test isolates from *Psoralea* species differs with nitrate nutrition. Feeding these strains with 59.3 mM nitrate results in significantly decreased concentration of lumichrome and riboflavin in cellular exudates, indicating an inhibitory effect of nitrate on the synthesis and release of the two metabolites. In fact, the levels of lumichrome in cell culture are decreased by high nitrate concentration for all the isolates. A similar decrease in Nod factor production has been observed by McKay and Djordjevic (1993), following nitrate supply to *Rhizobium leguminosarum* bv. *trifolii*. In the study by Kanu and Dakora (2012), it is interesting to note that the isolates, which showed the least production of riboflavin at high nitrate [e.g., TUT10pm and TUT13pac], are also among the least in lumichrome production at high nitrate. More importantly, however, the observed nitrate inhibition of lumichrome and riboflavin biosynthesis/release is in

addition to its known depressive effect on nodulation and N_2 fixation in symbiotic legumes (Streeter, 1988; Ayisi et al., 2000).

The observed variation in the secretion of lumichrome, riboflavin, and indole acetic acid by *Psoralea* root nodule isolates either exposed to different pH, salinity, and temperature regimes, or fed different levels of P, nitrate, and ammonium, suggests that genes encoding these metabolites are regulated differently by the imposed environmental factors (Kanu and Dakora, 2012). The data further suggest that natural changes in pH, salinity, and/or temperature in plant rhizospheres could potentially elevate the concentrations of lumichrome, riboflavin, and indole acetic acid in soils, with consequences for ecosystem functioning as both lumichrome and riboflavin have been reported to act as developmental signals that affect species in all three plant, animal, and microbial kingdoms.

38.6 ECOLOGICAL SIGNIFICANCE OF RHIZOBIAL EXUDATION OF LUMICHROME AND RIBOFLAVIN IN THE RHIZOSPHERE

In both natural and agricultural ecosystems, low or high production of lumichrome and riboflavin could have ecological consequences. For example, an increase in root respiration induced by lumichrome and riboflavin from root-colonizing rhizobia would lead to an elevated concentration of rhizosphere CO_2, which is needed for growth of rhizobial populations in soil (Lowe and Evans, 1962). Rhizobia and nodule endophytes isolated from eight *Psoralea* species (namely, *Psoralea pinnata*, *P. aphylla, P. aculeata*, *P. monophylla*, *P. repens, P. laxa*, *P. asarina*, and *P. restioides*) exhibit marked differences in the exudation of lumichrome, riboflavin, and indole acetic acid (Kanu and Dakora, 2009, 2012), three symbiotic signal molecules needed in bacteria–plant interactions (Phillips et al., 1999; Lambrecht et al., 2000; Matiru and Dakora, 2005a,b). Because the bacteria were isolated from different locations (e.g., Arabella Country Estate, Kleinmond, Betty's Bay, Rock Dam Valley, Kogelberg Nature Reserve, and Rooiles Nature Reserve) with differing soil conditions (e.g., wetland, upland), it is possible that their levels of metabolite secretion could be an adaptation to the local niche. As a result of their adaptation to the saline conditions of the beach, two *P. repens* strains isolated close to the ocean can secrete large amounts of lumichrome and riboflavin at both low and high salinities (Kanu and Dakora, 2009). Similarly, *Psoralea* isolates adapted to the acidic soils of the fynbos can produce greater amounts of indole acetic acid for symbiotic functioning even under very low pH conditions.

Furthermore, the increase in rhizosphere CO_2 concentration from lumichrome and riboflavin can stimulate growth of vesicular-arbuscular fungi (Becard and Piche, 1989; Becard et al., 1992) and increase the incidence of mycorrhizal symbiosis. These indirect benefits of lumichrome and riboflavin to legume symbioses via their effects on the plant are essential to enhance N and P nutrition. Also, the mere fact that most root-colonizing bacteria commonly produce and release lumichrome and riboflavin (Phillips et al., 1999), and some bacteria also produce eight times more extracellular riboflavin in exudates relative to internal cellular concentration suggesting that these molecules have evolved directly or indirectly as rhizosphere signals influencing outcomes of plant–bacterial interactions. The data further suggest that natural changes in pH, salinity, and/or temperature in plant rhizospheres could potentially elevate the concentrations of lumichrome, riboflavin, and indole acetic acid in soils, with consequences for ecosystem functioning as both lumichrome and riboflavin have been reported to act as developmental signals that affect species in all three plant, animal, and microbial kingdoms. The higher lumichrome production at lower temperature (10 °C) than at high temperature (30 °C) (Kanu and Dakora, 2009) has also ecological consequences for nodulation and N_2 fixation of *Psoralea* species in their Mediterranean fynbos habitat where winter rains support legume plant growth and symbiotic performance.

38.7 CONCLUSION

Bacterial exudation of the rhizosphere signals, lumichrome and riboflavin, can vary with rhizobial strain, soil temperature, and pH. Lumichrome taken up by plant roots and transported to the shoot probably elicits cell division, cell expansion, and cell extensibility, leading to an increased leaf expansion and stem elongation. A direct correlation exists between plant response to rhizobial inoculation and root application of lumichrome with respect to stomatal function. Both treatments consistently increase or decrease stomatal conductance and transpiration rates in responsive test species. Plant roots seem to be capable of collecting environmental signals from soil in the form of simple organic molecules released by microbes, and using them to adapt to their environment, for example, for drought tolerance when these molecules cause decreased stomatal conductance and consequently reduced water loss.

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Chapter 39

Genes Involved in Desiccation Resistance of Rhizobia and Other Bacteria

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39.1 INTRODUCTION

Rhizobia are Gram-negative, soil Alphaproteobacteria that are able to grow as free-living bacteria and can also form nitrogen-fixing, symbiotic relationships with legumes by inducing the formation of nodules on the roots of the plants. In these nodules, the bacteria convert dinitrogen gas from the atmosphere into ammonia, a form of nitrogen that can be used by the plant. To support this, the plant supplies the bacteria with carbon sources that are catabolized to provide the energy and reductant needed for nitrogen fixation (White et al., 2007). Formation of a nodule occurs after rhizobia in the soil infect the emerging root hairs of the plant. To survive when not in a symbiotic relationship, rhizobia must be able to cope with the conditions found in the soil or rhizosphere, including suboptimal pH and low water potential. Resistance to desiccation is not only an important phenotype for "wild" rhizobia living in the soil, but is also important in preparing rhizobia to be used as supplementary inocula in agricultural applications. Some rhizobia strains, such as *S. meliloti* Rm1021, are relatively desiccation resistant. After 2 months, 33% of Rm1021 cells were still viable after drying and subsequent rehydration (Humann et al., 2009). For other strains, such as *R. leguminosarum* 3841, only 37% of the cells are still viable after 16 h of drying (Gilbert et al., 2007). Figure 39.1 shows the differences in desiccation tolerance of four *S. meliloti* strains (J. L. Humann and M. L. Kahn, unpublished data). *S. meliloti* 2011 (Meade and Signer, 1977) and Rm104A14 (Somerville and Kahn, 1983) were much more desiccation resistant than Rm1021 (Meade et al., 1982) or its *expR*$^+$ derivative SM8530 (Pellock et al., 2002). Differences in desiccation tolerance among *B. japonicum* strains have also been observed (Osa-Afiana and Alexander, 1982b). Spore or cyst formation by rhizobia has not been observed (Jensen, 1961), suggesting that rhizobia have alternate adaptive mechanisms for surviving harsh environmental conditions. Vriezen et al. (2012; see Chapter 96) determined that during long periods of desiccation, a large portion of a *S. meliloti* 1021 cell population was viable but not culturable. The cells were intact, but did not form colonies when cultured. Desiccation research in rhizobia and other bacteria is starting to provide clues to the genes and pathways involved in desiccation resistance.

39.2 DESICCATION OF RHIZOBIA

Most of the early research on rhizobial desiccation resistance was physiologically based and was primarily directed at improving inocula and thus focused on what environmental factors and carrier materials could improve the survival of rhizobia prepared for agricultural applications (Bushby and Marshall, 1977; Chao and Alexander, 1982; Chao and Alexander, 1984; Kosanke et al., 1992; Osa-Afiana and

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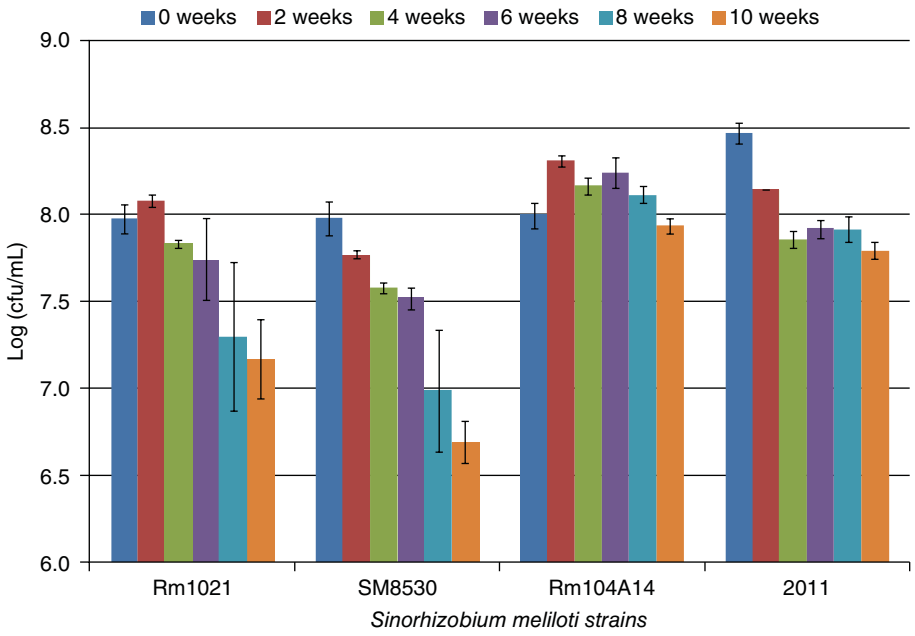

Figure 39.1 Survival of *Sinorhizobium meliloti* strains Rm1021, SM8530, Rm104a14, and 2011 to desiccation. The number of viable cells that survived desiccation was measured at 2 week intervals. Rm1021 and SM8530 had significant loss of viability, while Rm104a14 and 2011 showed less sensitivity to desiccation.

Alexander, 1982a; Vincent et al., 1962). Peat is the most common carrier for rhizobia inocula, but various materials have been used as carriers including coconut shells, charcoal, manure, and wheat straw (Chao and Alexander, 1984). Bushby and Marshall (1977) found that amending the soil with montmorillonite, maltose, sucrose, polyvinylpyrrolidone, or polyethylene glycol increased the survival rate of rhizobia under desiccating conditions. The effect of relative humidity on the desiccation tolerance of *Sinorhizobium meliloti* and *Bradyrhizobium japonicum* strains has been well documented (Mary et al., 1985; Mary et al., 1994). Although higher relative humidity is best for survival of rhizobia, if drying occurs at a slow pace the survival rate of rhizobia is higher than with rapid drying. Studies by Boumahdi et al. (1999; 2001) observed changes in cellular fatty acids during desiccation of *Sinorhizobium* and *Bradyrhizobium*. Short-chain fatty acids increased under reduced water activity and mild desiccation conditions. Vriezen et al. (2006) examined the effect of various physiological and physical conditions on the survival of *S. meliloti* during desiccation. They found that cells dried during the exponential growth phase had a lower survival rate than cells in a stationary growth phase at the time desiccation stress was initiated. Drying *S. meliloti* at temperatures above 37 °C also decreased the survival rate as did drying the cells on alfalfa seeds instead of on nitrocellulose or sand (Vriezen et al., 2006; Vriezen et al., 2012; see Chapter 96). Many factors affect the desiccation tolerance of rhizobia, and these factors make it difficult to replicate the natural desiccating conditions and the resulting bacterial response in the laboratory.

The goal of desiccation tolerance research is to determine how vegetative cells that are dried to a point where all biological processes have stopped survive extended periods of desiccation, and how the biological consequences of desiccation are resolved as the bacteria rehydrate. The desiccation process and response in prokaryotes has been reviewed in great detail (Billi and Potts, 2002; Potts, 1994; Potts, 2013). There are three stages of the desiccation process (Potts, 2013; Vriezen et al., 2007). Stage I is the drying of the cells, which results in the loss of water and the accumulation of salts and solutes and leads to osmotic and mechanical stress. Metabolic processes are also slowed and eventually stop due to lack of water. The lack of water may also contribute to increased DNA damage since the enzymes that would usually repair DNA and proteins are slowed or stopped. Stage II is the storage phase where the cells have lost all water and are just waiting to be rehydrated. Stage II is usually where the decline of cell viability occurs. The final step, stage III, is the rehydration of the cells. During this phase, bacterial metabolism is revived and enzymes repair the cell damage.

39.3 DESICCATION RESISTANCE GENES IN NONRHIZOBIA

Sensitivity to desiccation is a very common problem for bacteria, especially those that do not form spores. It is not clear whether similar or homologous mechanisms and genes are used to resist desiccation, but by examining all the available data common trends can be seen. DNA damage is an early effect of desiccation stress and two common types of DNA damage have been observed in desiccated bacterial cells: covalent modification and double-stranded breaks. After decades of desiccation, the DNA of the cyanobacteria *Nostoc commune* was covalently modified and formed

insoluble aggregates, but upon further examination the DNA was not degraded and did not exhibit signs of increased oxidative damage (Shirkey et al., 2003). Double-stranded DNA (dsDNA) breaks, which are very detrimental to the survival of bacteria, have been observed in desiccated cells of *Escherichia coli* (Asada et al., 1979), *Mycobacterium smegmatis* (Pitcher et al., 2007), and *Deinococcus radiodurans* (Dose et al., 1991; Mattimore and Battista, 1996).

The observation of DNA modification and damage in desiccated bacterial cells led to the identification of DNA repair genes that are involved in desiccation resistance from a variety of bacterial species. In *M. smegmatis*, deletion mutants lacking *ku* and *ligD*, the genes needed for bacterial nonhomologous end-joining DNA repair, exhibited increased sensitivity to desiccation (Pitcher et al., 2007). Desiccation-induced DNA damage has been studied in more detail in *D. radiodurans*, which is extremely resistant to ionizing radiation. The mechanisms needed for radioresistance and desiccation resistance somewhat overlap in this bacterium. Mattimore and Battista (1996) demonstrated that the longer wild-type *D. radiodurans* cells were dried, the more dsDNA breaks accumulated. A microarray study in *D. radiodurans* identified 32 upregulated genes that were induced in cultures recovering from desiccation or from ionizing radiation (Tanaka et al., 2004). Among these genes were common DNA repair genes such as *recA*, *ruvB*, *uvrA*, and *uvrB*. However, five other genes were the most highly expressed. Two of the genes (*ddrA* and *pprA*) encode DNA repair functions, *ddrB* encodes a single-stranded DNA-binding protein, and the remaining three genes (*ddrB*, *ddrC*, and *ddrD*) encode for proteins of unknown function (Harris et al., 2004; Narumi et al., 2004; Norais et al., 2009; Tanaka et al., 2004). RecA was also found to contribute to desiccation resistance in *Acinetobacter baumannii* (Aranda et al., 2011).

Regulatory and stress response genes, in addition to genes for biosynthesis of osmoprotectants and protein degradation, have also been implicated as having a role in desiccation resistance. Genes involved in stress response regulation (*sigB*) and protein degradation (*clpX* and *yjbH*) were found to have a role in desiccation resistance of *Staphylococcus aureus* (Chaibenjawong and Foster, 2011). In *Methylobacterium extorquens* AM1, PhyR is essential for plant surface colonization and is a response regulator that regulates several stress responses. A *phyR* deletion mutant was more sensitive to desiccation than the wild-type strain (Gourion et al., 2008). A transcriptomic analysis (LeBlanc et al., 2008) of desiccated *Rhodococcus jostii* RHA1 cells identified 406 genes that were upregulated during desiccating conditions (20% vs 100% RH). Among the mostly highly expressed transcripts were genes encoding stress response sigma factors (*sigF1*, *sigF2*), proteins that protect DNA during starvation (*dps1*, *dps2*) and two proteins in the ectoine biosynthesis pathway (*ectA*, *ectC*). Ectoine is a compound that helps bacteria survive osmotic stress by acting as an osmolyte. Trehalose production was linked to increased desiccation resistance in *E. coli* found in the soil (Zhang and Yan, 2012). Trehalose is an alpha-linked disaccharide that has a large capacity to retain water, which makes it a good compound to have around during desiccating conditions. In nonrhizobia, genes involved in DNA repair and protein degradation as well as genes that encode regulatory proteins and produce osmoprotectants have a role in desiccation resistance.

39.4 DESICCATION RESISTANCE GENES IN RHIZOBIA

Similar to other bacteria, the identified desiccation resistance genes in rhizobia also encode proteins involved in DNA repair, stress response, and osmoprotection. Studies have ranged from looking at the global response to desiccation via microarray experiments to looking at the effect of mutating specific genes on survival to dessication. A microarray study in *Bradyrhizobium japonicum* USDA 110 measured gene induction under desiccation stress (Cytryn et al., 2007). In *B. japonicum*, 225 genes were induced during all periods of desiccation, with an additional 446 genes induced during longer periods of desiccation. Genes that were induced during desiccation were diverse, but included genes involved in trehalose synthesis, EPS formation, sigma factors, oxidative and heat stress response systems, and DNA repair and modification.

One DNA repair system has been found to have a role in desiccation resistance of *S. meliloti*, and several others do not appear to affect viability. *S. meliloti* Rm1021 deletion mutants with lesions in the *uvrA*, *uvrB*, and *uvrC* genes were desiccation sensitive (Humann et al., 2009). These genes encode the nucleotide excision repair (NER) pathway, which targets lesions that distort the DNA helix, such as UV-induced cyclobutane pyrimidine dimers (CPDs) and 6,4-photoproducts, or bulky adducts caused by the reaction of DNA with various chemicals such as 4-NQO (Van Houten, 1990; Van Houten et al., 2005). While it has not been shown directly, it is reasonable to propose that *S. meliloti uvrA*, *uvrB*, and *uvrC* mutants cannot repair large, desiccation-induced DNA adducts and the accumulation of these is fatal. Although this desiccation-induced DNA damage is proposed to be recognized by the NER pathway, the exact nature of the DNA lesions is unknown. DNA from desiccated *S. meliloti* strains (wild-type and *uvr* mutants) was tested with CPD-specific antibodies (Gillette et al., 2001), and it was shown that these lesions were not present in the DNA of dried *S. meliloti* (J. L. Humann and M. L. Kahn, unpublished data). This is not surprising since CPDs and 6,4-photoproducts are typically generated by exposure of DNA to UV light, not desiccation. Deletion mutants

lacking *addAB*, *aidB*, *mutL*, *mutY*, *recA*, *ruvB*, *uvrD1*, or *uvrD2* were screened for desiccation sensitivity, and all the mutants had survival curves similar to that of the wild-type parent (J. L. Humann and M. L. Kahn, unpublished data). These data suggest that the *uvrABC* genes have a specific role in desiccation resistance, and that not all DNA repair mechanisms contribute to this type of cell durability.

Genes involved in cell membrane structure and exopolysaccharide (EPS) production have been identified as having a role in desiccation resistance in rhizobia. CtpA is a periplasmic protease that degrades misfolded periplasmic proteins, and a *ctpA* mutant in *Rhizobium leguminosarum* biovar *viciae* 3841 was 21 times more sensitive to desiccation than the wild type (Gilbert et al., 2007). The accumulation of defective proteins in the *R. leguminosarum ctpA* mutant cell envelope resulted in increased sensitivity to desiccation. Lipopolysaccharide (LPS) synthesis gene (*fabF1*, *fabF2*) mutants of *R. leguminosarum* biovar *viciae* 3841, which have modified very long-chain fatty acids, were impaired in the ability of the mutants to withstand desiccation (Vanderlinde et al., 2009). The EPS composition of the cell membranes also has an effect on desiccation resistance. In *Rhizobium sullae*, the production of high molecular weight EPS led to higher desiccation tolerance (Gharzouli et al., 2013). High molecular weight EPS compounds are very viscous and provide good osmoprotection. The importance of EPS was also observed in a desiccation-sensitive transposon mutant of *R. leguminosarum* biovar *viciae* 3841 (Vanderlinde et al., 2010). The transposon insertion was located in an ATP-binding protein that was part of an ATP-binding cassette transporter operon. The inactivation of the protein resulted in decreased EPS export and increased desiccation sensitivity.

In rhizobia, genes that encode stress response regulatory proteins have a role in desiccation resistance, but the exact role is harder to define. An *S. meliloti* Rm1021 transposon mutant with increased desiccation sensitivity had an insertion in the *relA* gene (Humann et al., 2009). RelA, a guanosine tetraphosphate synthetase and hydrolase, induces the stringent response during amino acid starvation (Wells and Long, 2002). In a *relA* mutant, the stringent response is not activated, and as a result the cell does not adjust to starvation quickly by downregulating protein synthesis and therefore depletes cellular metabolite pools more rapidly (Jensen and Pederson, 1990). In the case of desiccation, the lack of RelA activity appears to keep the cell from switching effectively to a "survival mode." A *relA* mutant of *Rhizobium etli* was also found to have decreased survival after heat shock, salt stress, or exposure to hydrogen peroxide (Braeken et al., 2008), further supporting the role of RelA in stress responses. A mutant in the *hpr* gene of *S. meliloti* also resulted in increased sensitivity to desiccation (Humann et al., 2009). HPr is a regulator of succinate-mediated catabolite repression (SCMR) in *S. meliloti* (Arango Pinedo et al., 2008). An *hpr* mutant in *S. meliloti* Rm1021 had altered carbon catabolism, lower survival in stationary phase, and early production of low molecular weight succinoglycan. Some or all of these physiological changes may contribute to decreased desiccation resistance. The homolog of *S. meliloti hpr* in *B. japonicum*, *ptsH*, is upregulated during desiccation, indicating that *hpr* may be involved in the drying stage of desiccation (Cytryn et al., 2007). Because these regulatory proteins affect so many different pathways, stress responses, and genes, it is hard to identify the exact role they play in desiccation resistance.

It is logical that stress response sigma factors would have a role in desiccation resistance. The *S. meliloti rpoE2* gene, which encodes a sigma factor that regulates extracytoplasmic function, is upregulated during salt stress, entry into stationary phase after carbon or nitrogen starvation, and heat shock (Sauviac et al., 2007; see Chapter 40). In addition, genes induced during desiccation in *B. japonicum* USDA 110 include an *rpoE* gene (Cytryn et al., 2007). The homolog of RpoE2 of *B. japonicum* USDA 110, *ecfG*, is also involved in heat shock and desiccation resistance during carbon starvation (Gourion et al., 2009). A *rpoE2* transposon mutant in *S. meliloti* Rm1021 was found to be sensitive to desiccation (Humann et al., 2009). The *M. extorquens* AM1 *phyR* gene described earlier is also involved in desiccation resistance of rhizobia. In *B. japonicum*, a *phyR* mutant had increased sensitivity to desiccation along with defects in nodulation and nitrogen fixation (Gourion et al., 2009). The *S. meliloti* Rm1021 *phyR* homolog is known as *rsiB1*. RsiB1 is an anti-anti-sigma factor that actually allows for the expression of *rpoE2* during stress conditions such as heat-shock and stationary phase (Bastiat et al., 2010; see Chapter 40). An *S. meliloti* Rm1021 *rsiB1* deletion mutant was more sensitive to desiccation than the wild type, indicating that the lack of *rpoE2* expression due to the *rsiB1* mutation is similar to having an *rpoE2* mutation (J. L. Humann and M. L. Kahn, unpublished data). The RpoE2 sigma factor has also been linked to trehalose accumulation, which has been implicated in desiccation resistance of rhizobia. An *otsA* (trehalose-6-phosphate synthase) and *treY* (maltooligosyltrehalose synthase) double mutant of *R. leguminosarum* bv. *trifolii*, which did not accumulate trehalose, exhibited increased sensitivity to drying (McIntyre et al., 2007). In *S. meliloti*, *otsA* expression is induced by the stress response sigma factor RpoE2 (Flechard et al., 2010). As a result, the desiccation-sensitive phenotype of the *S. meliloti rpoE2* mutant (Humann et al., 2009) may be related to a lack of trehalose synthesis and accumulation. As with the RelA and Hpr, the role that RpoE2 plays in desiccation resistance is potentially complicated because these proteins regulate various physiological processes that could act in desiccation resistance.

Table 39.1 Genes that contribute to desiccation resistance in bacteria as determined by mutation or deletion

Cellular Process	Genus and Species	Gene Name or ID	Function	Reference
DNA repair	*Mycobacterium smegmatis*	*ku*	Nonhomologous end-joining repair	Pitcher et al. (2007)
	Mycobacterium smegmatis	*ligD*	Nonhomologous end-joining repair	Pitcher et al. (2007)
	Acinetobacter baumannii	*recA*	DNA recombination and repair	Aranda et al. (2011)
	Sinorhizobium meliloti	*uvrA*	Nucleotide excision repair (NER) pathway	Humann et al. (2009)
	Sinorhizobium meliloti	*uvrB*	Nucleotide excision repair (NER) pathway	Humann et al. (2009)
	Sinorhizobium meliloti	*uvrC*	Nucleotide excision repair (NER) pathway	Humann et al. (2009)
Stress responses	*Bradyrhizobium japonicum*	*ecfG*	Extracytoplasmic function (ECF) type sigma factor	Gourion et al. (2009)
	Sinorhizobium meliloti	*hpr*	Succinate mediated catabolite repression (SCMR) regulator	Humann et al. (2009)
	Bradyrhizobium japonicum	*phyR*	Stress response regulator	Gourion et al. (2009)
	Methylobacterium extorquens	*phyR*	Stress response regulator	Gourion et al. (2008)
	Sinorhizobium meliloti	*relA*	Stringent response regulator	Humann et al. (2009)
	Sinorhizobium meliloti	*rpoE2*	Extracytoplasmic function (ECF) type sigma factor	Humann et al. (2009)
	Sinorhizobium meliloti	*rsiB1*	Regulates *rpoE2* expression	Humann and Kahn, unpublished
	Staphylococcus aureus	*sigB*	Stress-response alternative sigma factor	Chaibenjawong and Foster (2011)
Protein structure	*Rhizobium leguminosarum*	*ctpA*	Periplasmic protease	Gilbert et al. (2007)
	Staphylococcus aureus	*clpX*	ATP-dependent ClpXP protease	Chaibenjawong and Foster (2011)
	Staphylococcus aureus	*yjbH*	Protein degradation	Chaibenjawong and Foster (2011)
LPS/EPS production	*Rhizobium leguminosarum*	*fabF1*	Lipopolysaccharide (LPS) synthesis	Vanderlinde et al. (2009)
	Rhizobium leguminosarum	*fabF2*	Lipopolysaccharide (LPS) synthesis	Vanderlinde et al. (2009)
	Rhizobium leguminosarum	RL2975	ATP-binding protein of ABC transporter	Vanderlinde et al. (2010)
Osmoprotectant production	*Rhizobium leguminosarum*	*otsA*	Trehalose accumulation	McIntyre et al. (2007)
	Rhizobium leguminosarum	*treY*	Trehalose accumulation	McIntyre et al. (2007)

39.5 SUMMARY

Desiccation tolerance by bacteria is a multifaceted process that involves DNA repair, stress response induction, membrane structure, and the production of osmoprotectant compounds. Table 39.1 summarizes all the genes that have been confirmed by mutation or deletion to be involved in bacterial desiccation resistance. This table does not include the hundreds of genes that were upregulated or downregulated in microarray studies of desiccated bacterial cells. Desiccation tolerance and the cellular processes that are involved appear to have similarities among all bacteria. While desiccation-induced DNA damage has been extensively studied in *D. radiodurans* (Mattimore and Battista, 1996; Tanaka et al., 2004) and numerous DNA repair genes have been identified, there appears to be some differences in the DNA repair genes that are involved in desiccation. Although RecA is needed for desiccation resistance in *M. smegmatis* (Pitcher et al., 2007), a *recA* mutant in *S. meliloti* was not more sensitive to desiccation (J. L. Humann and M. L. Kahn, unpublished data). The question also remains about what type of desiccation-induced DNA damage the NER pathway repairs in *S. meliloti*. A role for the stress-response sigma factors and sigma factor regulators in desiccation resistance has been observed in rhizobia (see Chapter 40) as well as in *M. extorquens* (Gourion et al., 2008) and *S. aureus* (Chaibenjawong and Foster, 2011). However, since hundreds of genes can be regulated by a sigma factor, these mutations are not very specific in identifying specific processes that contribute to desiccation resistance. It is very clear that there is not just one operon or biological process that contributes to desiccation resistance, and instead a number of genes deal with different aspects of changes caused by the lack of water and ultimately of normal metabolic activity (see also Chapter 96).

The different levels of desiccation resistance within the same species of rhizobia or between different genera of rhizobia raise interesting questions about how the life history of these bacteria might differ. The differences are also important for the agricultural application of rhizobial inocula. If all rhizobia were highly tolerant to desiccation, their use as inocula for legume crops would be more cost effective and biologically effective. Are the differences due to expression levels of the genes identified in Table 39.1? Or is it because some rhizobia lack certain genes that are involved in desiccation resistance? Experiments looking for the presence and absence of genes or at differences in gene expression levels during desiccation challenges among the rhizobia might provide clues about how to engineer rhizobia to be more desiccation resistant and therefore more suitable for agricultural applications.

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Chapter 40

The General Stress Response in Alpha-Rhizobia

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40.1 INTRODUCTION

Bacteria are exposed to constantly changing environmental conditions in nature, which can be stressful and limit their growth, or even lead to cell death. Bacteria have therefore evolved adaptation responses that make them able to cope with these fluctuations. Some of the responses function by eliminating the inducing stress and/or repairing the associated cell damages. In parallel to these stress-specific responses, a so-called general stress response is activated under many different stress or starvation conditions, resulting in a number of morphological, physiological, and metabolic changes that confer multiple stress resistance to the cells. Essential features of this response include cross-protection and prevention, since bacteria exposed to one particular stress become resistant not only to this inducing stress but also to other stresses that they have not experienced yet. The general stress response therefore appears as both short- and long-term adaptation responses, particularly in the absence of growth, a prevailing situation in nature where nutrients are often limiting (Hengge, 2011). Studies on model bacteria have revealed that the general stress response mainly relies on a global reorganization of gene expression, in which sigma factors play a central role. Sigma factors are dissociable subunits of the RNA polymerase that confer the specificity of gene promoter recognition. In bacteria growing actively without stress, the vegetative sigma factor is responsible for the transcription of housekeeping genes. Under stress conditions, various signals and mechanisms make new sigma factors available for interacting with the core RNA polymerase, thus allowing the holoenzyme to recognize new promoters and express new sets of genes (Sterberg et al., 2011). In *Bacillus subtilis* and other Gram-positive bacteria, the general stress response is controlled by σ^B (Hecker et al., 2007; Price, 2011), whereas in *Escherichia coli* and related γ-Proteobacteria, as well as several β- and δ-Proteobacteria, it is governed by σ^S (Battesti et al., 2011; Hengge, 2011). In all cases, activation of the sigma factors makes possible the expression of hundreds of new genes, some of which are involved in the acquisition of multiple stress resistance.

Rhizobia are soil bacteria that can enter a nitrogen-fixing symbiotic association with legume plants. In their natural environments, these bacteria are exposed to many stressful conditions that threaten their survival in the soil and, directly or indirectly, the efficiency of symbiotic nitrogen fixation. Among stresses that rhizobia can encounter in the soil are high and low temperatures, salinity, desiccation, low or high pH, as well as nutrient and oxygen deprivation (Zahran, 1999; van Veen et al., 1997). In plant tissues, bacteria are also exposed to oxidative and nitrosative stress conditions (Santos et al., 2001, Baudouin et al., 2006). A number of stress-specific responses have been described in rhizobia, leading to the identification of specific proteins and regulators involved in these responses (see for instance Meilhoc

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et al., 2010; Jamet et al., 2005; Sagot et al., 2010; Minder et al., 2000). In contrast, until recently, much less was known about the general stress response and its regulation in these bacteria. Only a few of the known rhizobia (those belonging to the β-subclass of Proteobacteria) encode a σ^S homolog (Amadou et al., 2008), likely involved in the general stress response of these bacteria (although this has not been investigated so far). However, most of the known rhizobia are α-Proteobacteria (the so-called α-rhizobia), and none of the sequenced genomes of these bacteria encode a σ^S homolog. We review here the advances made in recent years in our understanding of the general stress response in α-rhizobia, its regulation, and its functions.

40.1.1 Evidence of the Existence of a General Stress Response in α-Rhizobia

Under laboratory conditions, the general stress response is activated when bacteria enter into the stationary phase. Thus, stationary-phase bacteria usually display a better resistance to multiple stresses as compared to exponentially growing bacteria. In *Rhizobium leguminosarum*, stationary-phase bacteria have been found to be more resistant than exponentially growing bacteria to heat, salt, oxidative, and acidic stresses (Thorne and Williams, 1997). Similarly, in *Sinorhizobium meliloti*, stationary phase bacteria have been found to be more resistant than exponentially growing bacteria to desiccation, oxidative, and heat stresses (Vriezen et al., 2006; see Chapter 96, Fléchard et al., 2009; Barra-Bily et al., 2010). In *Bradyrhizobium japonicum*, bacteria allowed to spend one night under carbon starvation have been found to be more heat resistant than bacteria challenged in the exponential phase of growth (Gourion et al., 2009). Taken together, these observations suggest that a general stress response of the same type as that described in other bacteria does indeed exist in certain rhizobia.

Another characteristic feature of the general stress response is its induction by diverse stress conditions. Pioneer studies aiming at the identification of genes upregulated under various conditions were first conducted on *S. meliloti* using reporter fusion libraries (Milcamps et al., 1998), but the most comprehensive studies have more recently been carried out using transcriptomic approaches. For instance, 60 *S. meliloti* genes have been found to be upregulated either after heat treatment or upon entry in stationary phase following carbon exhaustion from the culture medium (Sauviac et al., 2007). Expression of many of these genes has also been found to be induced under various other conditions, including microaerobiosis (Bobik et al., 2006; Becker et al., 2004), hyperosmotic conditions (Domínguez-Ferreras et al., 2006), and acidic pH (de Lucena et al., 2010), which suggests that they are part of the general stress response of this rhizobium species. Sauviac et al. (2007) noticed that one of these genes (*rpoE2*) encodes an extracytoplasmic function (ECF) sigma factor. RpoE2 turned out to be activated in these diverse conditions and to control the transcription of a large part of the "general stress response genes" identified (Sauviac et al., 2007). Interestingly, this sigma factor belongs to the ECF15 subgroup of ECF sigma factors (also called EcfG) specifically found in α-Proteobacteria where it is largely conserved, including in most α-rhizobia (Staroń et al., 2009). Subsequent studies conducted in some of these species (*Methylobacterium extorquens*, *Caulobacter crescentus*, *B. japonicum*, *Rhizobium etli*) have confirmed the involvement of EcfG sigma factors in the transcriptional control of large regulons in response to various external stresses (Alvarez-Martinez et al., 2007; Gourion et al., 2008; Gourion et al., 2009; Martínez-Salazar et al., 2009a; see Chapter 30 for a review see Francez-Charlot et al., 2011). In addition, in all these species, *ecfG* mutants have been found to be more sensitive than the wild-type strain to many stress conditions, including osmotic, oxidative, and thermic stresses (see later). Altogether, these observations indicate that EcfG sigma factors play the function of regulators of the general stress response and represent the long searched for functional analogs of the σ^S and σ^B described in model bacteria. In the following sections, we summarize the features of EcfG sigma factors, their regulation in response to stress, and the known functions of the response, with a special focus on α-rhizobia.

40.2 σ^{EcfG}, THE MAJOR REGULATOR OF THE GENERAL STRESS RESPONSE

40.2.1 The Basic σ^{EcfG} Transduction Cascade in α-Proteobacteria

A number of genetic, biochemical, and structural approaches have been conducted in various bacteria in order to decipher the signaling pathway of σ^{EcfG} activation in response to stress (Sauviac et al., 2007; Francez-Charlot et al., 2009; Gourion et al., 2009; Bastiat et al., 2010; Herrou et al., 2010; Lourenço et al., 2011; Kaczmarczyk et al., 2011; Herrou et al., 2012; Campagne et al., 2012; Sauviac and Bruand, submitted). The basic mechanism seems to be conserved in most α-Proteobacteria, with species-specific variations. It primarily involves three proteins: the sigma factor, an anti-sigma factor, and an anti-antisigma factor. All three components are almost always encoded by genes colocalized in the same genomic locus. The distribution and genomic organization of these genes in various α-rhizobia is shown in Figure 40.1.

The current model of σ^{EcfG} regulation is schematically depicted in Figure 40.2. In the absence of stress, the sigma factor is kept inactive by interaction with the anti-sigma

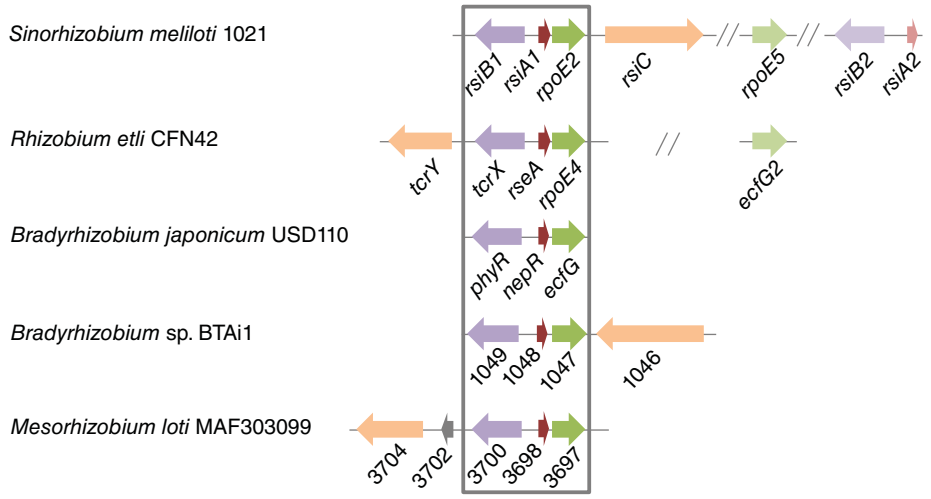

Figure 40.1 Genomic organization of genes known or supposed to be involved in the σ^{EcfG} transduction cascade in selected alpha-rhizobia. The conserved *ecfG* loci, encoding the main EcfG sigma factor (green), the anti-sigma (red), and the anti-antisigma (purple) are boxed in gray. Secondary genes encoding additional sigma, anti-sigma, or anti-antisigma factors are indicated when present, their genomic independency from the main locus being symbolized by //. Putative histidine kinases, when present in the vicinity of the conserved locus, are depicted in orange (note that only *S. meliloti* RsiC was shown to be involved in the control of the phosphorylation status of RsiB so far (Sauviac and Bruand, submitted)). A similar organization as in *S. meliloti* 1021 was found in other *S. meliloti* strains (AK83, 2011, BL225C, SM11, RM41, and GR4) and in *S. medicae* WSM419 and *S. fredii* (strains HH103, USDA277, and NGR234). A similar organization as in *R. etli* CFN42 was found in *R. etli* CIAT652, *R. tropici* CIAT899, *R. leguminosarum* bv. *viciae* 3841, and *R. leguminosarum* bv. *trifolii* (strains WSM1325 and WSM 2304), except that the latter three strains encode 0, 2, and 3 secondary *ecfG* genes, respectively. A similar organization as in *B. japonicum* USDA110 was found in *B. japonicum USDA6*. A similar organization as in *Bradyrhizobium* BTAi1 was found in *Bradyrhizobium* ORS278. A similar organization as in *M. loti* MAFF303099 was found in *M. ciceri* bv. biserrulae WSM1271, *M. australicum* WSM2073, and *M. opportunistum* WSM2075.

factor, called RsiA in *S. meliloti* or NepR in *B. japonicum* (see Chapter 30). This anti-sigma factor is a small (~60 amino acids) and seemingly soluble, cytoplasmic protein. Under stress conditions, a third protein, called RsiB or PhyR, respectively, becomes activated. RsiB/PhyR is an atypical response regulator of two-component regulatory systems, composed of two domains. The C-terminal domain carries a phosphor-receiver domain, with a conserved phosphorylatable aspartate residue, while the N-terminal domain shows homologies to ECF sigma factors. Upon stress exposure, the conserved aspartate residue of the receiver domain is phosphorylated, thus leading to a conformational change which releases the N-terminal domain. Because of its structural similarity with the sigma factor and its high affinity for the anti-sigma, this domain enters in competition for binding the anti-sigma, thereby releasing the sigma factor, which then becomes available for interacting with the core RNA polymerase and directing the transcription of its target genes. The sigma, anti-sigma, and anti-antisigma factor-encoding genes are all part of the σ^{EcfG} regulons, thus providing both positive- and negative-feedback regulatory loops to the system.

40.2.2 Signal Perception and Activation of the σ^{EcfG} Transduction Cascade

How PhyR/RsiB becomes phosphorylated under stress conditions is still not always completely clear. However, and not surprisingly, given the typical phosphor-receiver domain of the anti-antisigma, histidine kinases have been implicated in this reaction in *C. crescentus*, *Sphingomonas* sp. Fr1, and *S. meliloti* (Lourenço et al., 2011; Kaczmarczyk et al., 2011; Sauviac and Bruand, submitted). These proteins are encoded in the vicinity of their respective *ecfG* loci, but this organization is not always conserved, as seen, for example, in *B. japonicum* (see Fig. 40.1). These proteins are atypical in that they do not contain the usual domain of dimerization and histidine phosphotransfer found in classical histidine kinases, but instead harbor either a Pfam:HWE_HK or, less frequently, a Pfam:HisKA_2 domain (Staroń and Mascher, 2010). The proteins investigated so far were involved in controlling the phosphorylation status of RsiB/PhyR, through activation of their kinase activity and/or inhibition of their phosphatase activity in the presence of stress (Lourenço

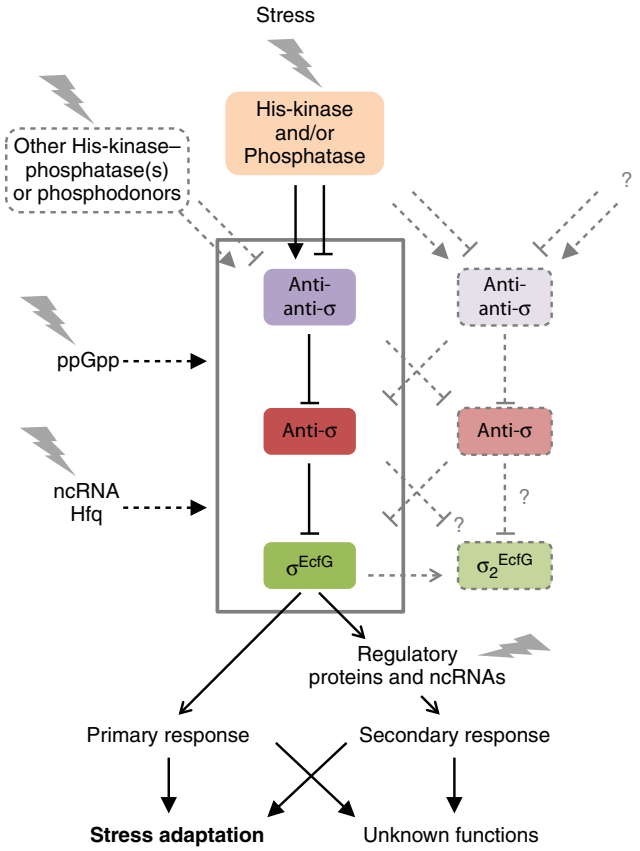

Figure 40.2 The σ^{EcfG} transduction cascade. The main conserved transduction cascade is boxed in gray, with the different proteins colored as their corresponding genes in Figure 40. 1. Arrows and T lines represent activations and inhibitions, respectively (open arrowheads: transcriptional activations, closed arrowheads: other types of activation). Possible variations of the cascade and other factors influencing the cascade are depicted as dotted arrows and proteins. Putative stress inputs are depicted as gray flashlights.

et al., 2011; Kaczmarczyk et al., 2011; Sauviac and Bruand, submitted). However, the nature of the inducing signals and how they are, directly or indirectly, perceived by these kinases/phosphatases, are still unknown. It is striking however that the N-terminal putative sensor domains of these proteins carry variable motifs, and that these proteins can be either cytoplasmic or membrane located, indicating that they can perceive various signals of diverse origins (Staroń and Mascher, 2010).

40.2.3 Variations Around the Basic Cascade

Variations to this basic model have been described in several bacteria (see Figs. 40.1 and 40.2). Additional (one or more) EcfG sigma factors often coexist in the same species, and are usually under the transcriptional control of the main EcfG sigma factor, although not encoded in a conserved genomic context (e.g., RpoE2 and RpoE5 in *S. meliloti*, or RpoE4 and EcfG2 in *R. etli*; Fig. 40.1). In *R. etli*, both sigma factors have been shown to be partially redundant for regulating the general stress response (Vercruysse et al., 2011). In *Sinorhizobium* species, two pairs of anti-sigma/anti-antisigma are encoded in the genome (RsiA1/B1 and RsiA2/B2), and in *S. meliloti*, are able to cross talk and cooperate to fully control sigma factor activity (Bastiat et al., 2010). In this case, however, expression of the second pair is not under the control of σ^{EcfG} and is therefore not sensitive to feedback regulatory control. In some species, additional histidine kinase/phosphatases of the same family and/or other phosphodonors have been proposed to cross talk under particular conditions for phosphorylating or dephosphorylating RsiB/PhyR, thus adding additional levels of control (Foreman et al., 2012; Sauviac and Bruand, submitted), although the biological significance of these findings remains to be established.

40.2.4 Other Factors Acting on the Transduction Cascade

Several reports suggest that still other factors are able to modulate the activity of this basic cascade (Fig. 40.2). In *R. etli*, expression of the RpoE4 regulon was shown to be downregulated in stationary phase in a *rsh* (*relA*) mutant, deficient in the synthesis of the alarmone (p)ppGpp (Vercruysse et al., 2011). Interestingly, this mutant is known to be more sensitive to heat, osmotic, and oxidative stress than the wild-type strain, and to be affected in stationary-phase survival (Braeken et al., 2008). This suggests that some of these phenotypes could result from a defect of the general stress response. In *E. coli* and other bacteria, (p)ppGpp is synthesized upon entry in stationary phase, and is known to increase the competitiveness of alternative sigma factors for binding the RNA polymerase, thereby increasing their contribution to gene expression in comparison with that of the vegetative sigma factor. In this manner, (p)ppGpp participates in the activation of alternative sigma factors in stationary phase, including the general stress response regulator σ^S and the ECF sigma factor σ^E (Sharma and Chatterji, 2010; Magnusson et al., 2005; Costanzo et al., 2008). The results obtained in *R. etli* therefore suggest that (p)ppGpp could be one of the factors contributing to the activation of the σ^{EcfG}-dependent general stress response in the stationary phase. In *S. meliloti*, however, no differential expression of the RpoE2 regulon was observed between wild-type and *relA* mutant bacteria upon entry in stationary phase (Krol and Becker, 2011), suggesting that the effect of (p)ppGpp on σ^{EcfG} activity is not universal. Alternatively, as *S. meliloti relA* mutants are known to accumulate suppressor mutations (Wells and Long, 2003), it cannot be ruled out

that the mutant strain used by Krol and Becker contains such a suppressor that restores normal RpoE2 activity.

In *S. meliloti*, the expression of *rpoE2* and some of its targets in the stationary phase was lowered in an *hfq* mutant. At the same time, the increase in stress resistance (heat, osmotic, and oxidative stresses) usually observed in stationary phase in the wild-type strain was impaired in the *hfq* mutant (Barra-Bily et al., 2010). These phenotypes could partly result from a deficiency of the general stress response. In *E. coli* and other bacteria, Hfq is an RNA chaperone involved in the activity of *trans*-acting noncoding small RNAs (sRNAs). sRNAs modulate gene expression through various mechanisms, including activation or inhibition of translation by Hfq-dependent base-pairing with RNA transcripts (Gottesman and Storz, 2011). In *E. coli*, σ^S translation is activated by several sRNAs with the help of Hfq (Repoila et al., 2003). On the other hand, the activity of σ^E is inhibited by two Hfq-dependent sRNAs whose expression is itself under σ^E control. This inhibition is indirect as the sRNAs inhibit the translation of outer membrane proteins whose presence in the periplasm triggers RpoE activation (Gogol et al., 2011). Thus, sRNAs, and by extension Hfq, can directly or indirectly affect the activity of sigma factors. In consequence, it is assumed that RpoE2 activity in *S. meliloti* is influenced directly or indirectly by one or more sRNAs. Interestingly, the RpoE2 regulon itself includes several sRNAs (see later), but their involvement in RpoE2 regulation has not been tested so far.

40.3 FUNCTIONS OF THE σ^{EcfG}-DEPENDENT GENERAL STRESS RESPONSE

40.3.1 The σ^{EcfG} Regulons

σ^{EcfG} regulons were determined in several α-Proteobacterial species, including three rhizobia: *S. meliloti*, *B. japonicum*, and *R. etli* (Sauviac et al., 2007; Gourion et al., 2009; Martínez-Salazar et al., 2009a; Schlüter et al., 2013). For this, microarrays or affymetrix chips were used to compare the transcriptomes of wild type and either *ecfG*-mutant or *ecfG*-overexpressor strains, grown under stress/starvation conditions or under exponential growth conditions. In *B. japonicum*, the PhyR regulon was also established by comparing the transcriptomes of wild-type and *phyR*-mutant strains, and has been found to be congruent with the σ^{EcfG} regulon, as expected since PhyR and σ^{EcfG} act in the same cascade (see earlier). These different analyses have led to the identification of ∼100–200 genes directly or indirectly regulated by the sigma factor. Interestingly, most genes were found to be induced by σ^{EcfG}, as expected from a transcriptional activator, whereas a few genes were found to be downregulated, suggesting either indirect σ^{EcfG} regulations or side effects of the *ecfG* mutations.

From all these studies, as well as from experimental mapping of transcription start sites, either for individual genes or at the genome scale using RNA sequencing approaches, it has been possible to identify a conserved motif in the upstream DNA regions of at least half of the σ^{EcfG}-dependent genes or operons: GGAAC-N(16-17)-CGTT, which presumably corresponds to the −35 and −10 regions recognized by σ^{EcfG}. Genes whose expression is regulated by σ^{EcfG} but do not carry such sequences in their promoter region are likely indirectly regulated by σ^{EcfG}. Conversely, a search for such a motif upstream from genes in whole sequenced genomes, or upstream transcription start sites in RNA-seq data has made the identification of additional putative σ^{EcfG} targets possible (Sallet et al., 2013; Schlüter et al., 2013). In all, ∼100–200 putative direct target promoters of σ^{EcfG} could be identified in each single genome, corresponding to at least 150–300 σ^{EcfG}-controlled genes, therefore confirming that these sigma factors are global regulators of gene expression, as expected for general stress regulators.

40.3.2 Indirect σ^{EcfG} Regulation

Another subset of σ^{EcfG} targets encodes putative regulators, which potentially make it possible to integrate additional signals and regulate additional sets of genes, thus forming a complex regulatory network. These regulators are likely responsible for the transcriptional control of some of the "indirect" σ^{EcfG} targets. Two kinds of regulators were identified: proteins and sRNAs. Regulatory proteins include sigma factors and one- or two-component transcriptional regulators. Of the sigma factors, one or several σ^{EcfG}-like factor(s) are often present. In *R. etli*, experimental evidence led to the suggestion that the two EcfG sigma factors control partly nonoverlapping sets of genes, but the genes preferentially regulated by either one of these sigma factors have not been identified (Vercruysse et al., 2011). Also, the transcription of one heat-shock sigma factor-encoding gene was found to be σ^{EcfG}-dependent (*rpoH2* in *S. meliloti* and *R. etli*), or suggested to be σ^{EcfG}-dependent, based on the stress inducibility and/or presence of upstream σ^{EcfG} binding sequences (see Sauviac et al., 2007 and references therein). How these rhizobial RpoH sigma factors are activated in response to stress is currently not known, and how their function differs from that of other RpoH sigma factor(s) present in these bacteria is presently unclear. Recent data have shown that *S. meliloti* RpoH2 does not play a significant regulatory role under heat shock conditions, but instead is active in stationary phase, and putative target genes have been identified (Barnett et al., 2012). Interestingly, some of the genes found to be upregulated upon RpoE2 overexpression are actually RpoH2 targets, which demonstrates that these sigma factors indeed act in a cascade (Schlüter et al., 2013).

Other σ^{EcfG}-dependent regulators include numerous members of two-component regulatory systems, the function of which is mostly unknown. In *S. meliloti*, two such systems appear under the control of RpoE2: SMa0113-SMa0114 and *exsF-exsG*. The former is involved in the regulation of succinate-mediated catabolite repression (Garcia et al., 2010), which could be consistent with a function of the σ^{EcfG}-dependent response during the transition from active growth to starvation conditions. The function of *exsF-exsG* is unknown, although its location in the middle of a cluster of *eps* genes may indicate a role in exopolysaccharide synthesis. Interestingly, both SMa0114- and *exsF*-encoded proteins belong to the same unusual family of histidine kinases as the kinases/phosphatases of the σ^{EcfG} transduction cascades (see earlier), pointing to an intriguing link between EcfG sigma factors and this protein family.

A number of sRNAs have been shown to be under direct control of RpoE2 in *S. meliloti* in two recent studies (Sallet et al., 2013; Schlüter et al., 2013). Additional sRNAs may also be indirectly controlled by σ^{EcfG}, such as the several *S. meliloti* sRNAs regulated by RpoH2 (Barnett et al., 2012; Schlüter et al., 2013). However, nothing is known to date about the possible targets and functions of these sRNAs in stress responses.

40.3.3 Phenotypic Analyses of Mutants in the σ^{EcfG} Cascade

Numerous phenotypic analyses have been conducted on mutants of the σ^{EcfG} transduction cascade in different α-Proteobacteria, leading to the definitive demonstration that these sigma factors are central regulators of the general stress response in these bacteria, playing a role analogous to that of σ^S and σ^B in *E. coli* and *B. subtilis*, respectively. Here, we mainly summarize the observations made in rhizobia.

40.3.3.1 Free-Living Phenotypes. Mutants of the sigma factor (*ecfG*) or the anti-antisigma (*phyR/rsiB/tcrX*) have been shown to display similar pleiotropic phenotypes in several rhizobia, that is an increased sensitivity to various stress conditions. In *R. etli*, the importance of the two EcfG-type sigma factors in stress resistance was highlighted by the fact that single *rpoE4* and *ecfG2* mutants are more sensitive than the wild-type strain to H_2O_2 or heat treatment, and that a double mutant is even more sensitive to these stresses (Vercruysse et al., 2011). Involvement of the secondary EcfG sigma factor RpoE5 in stress resistance of *S. meliloti* has not been tested so far.

It is noteworthy that, at least in *S. meliloti* and *B. japonicum*, stress sensitivity of *ecfG* mutants has been detected in bacteria from stationary-phase cultures, or those having spent one night under carbon starvation conditions, but not in exponentially growing bacteria (Barra-Bily et al., 2010; Fléchard et al., 2009; Gourion et al., 2009). This further supports the particular importance of the general stress response when bacteria are in stationary phase.

S. meliloti rpoE2 and *R. etli rpoE4*, *ecfG2*, and *tcrX* mutants have been found to be more sensitive to hydrogen peroxide than the isogenic wild-type strains (Martínez-Salazar et al., 2009a; Fléchard et al., 2009). In particular, *S. meliloti* has been shown to be more resistant to hydrogen peroxide in stationary phase than in exponential phase in an RpoE2-dependent manner. In both rhizobia, a catalase-encoding gene is controlled by the EcfG sigma factor. Thus, the *S. meliloti katC* gene is induced in stationary phase following exhaustion of either the carbon or nitrogen source, as well as in exponential phase following treatment with diverse stress agents (Jamet et al., 2003; Sauviac et al., 2007; Fléchard et al., 2009). Accordingly, the peroxydase activity of KatC was observed to increase in stationary phase (Jamet et al., 2003). Since a *katC* mutant has been found to phenocopy an *rpoE2* mutant, *katC* is supposed to be the gene responsible for RpoE2-dependent H_2O_2 resistance (Fléchard et al., 2009). One can assume that the CH00462 Mn-catalase controlled by RpoE4 plays a similar role in *R. etli*. Although catalases are probably the main actors of the EcfG-dependent H_2O_2 resistance, one cannot exclude the possibility that other genes are involved.

Among other σ^{EcfG}-dependent genes possibly involved in oxidative stress response are those that encode superoxide dismutases, such as the *sodC* genes in *S. meliloti* and *R. etli*. Although *R. etli rpoE4* and *tcrX* mutant cells are more sensitive to the superoxide generator methyl viologen than that of wild-type cells (Martínez-Salazar et al., 2009a), the *S. meliloti rpoE2* mutant was found to be as resistant as wild-type bacteria to external superoxide (Fléchard et al., 2009).

S. meliloti rpoE2 and *R. etli rpoE4*, *ecfG2*, and *tcrX* mutants have also been found to be more sensitive to osmotic stress than wild-type bacteria, as measured by sucrose and/or NaCl sensitivity, whereas *B. japonicum ecfG* and *phyR* mutants are not (Fléchard et al., 2010; Gourion et al., 2009). In *S. meliloti*, osmosensitivity of the *rpoE2* mutant could be associated with its deficiency in trehalose synthesis, a well-known osmoprotective compatible solute. The expression of genes involved in three different pathways of trehalose synthesis (*otsA*, *treS*, and *treY*), known to be required for osmoresistance (Domínguez-Ferreras et al., 2009), has been shown to be dependent on *rpoE2* in stationary phase and/or in exponential phase following a salt shock in *S. meliloti* (Fléchard et al., 2010). Similarly, in *B. japonicum*, expression of these genes, as well as the corresponding enzyme activities, increases *in vivo* in the presence of salt (Streeter and Gomez, 2006; Sugawara et al., 2010), and has been found to be dependent on σ^{EcfG} under carbon starvation conditions (Gourion et al., 2009).

Although trehalose synthesis genes obviously play a major role in σ^{EcfG}-associated osmoresistance, we cannot rule out the hypothesis that other genes are involved. Thus, *rpoH2*, which is often found among σ^{EcfG} targets, is involved in osmotic tolerance in *R. etli* and *Sinorhizobium* sp. BL3, suggesting that some RpoH2 targets are involved in osmotic resistance (Tittabutr et al., 2006; Martínez-Salazar et al., 2009b).

The *S. meliloti rpoE2* mutant was found to be more sensitive to heat stress than its wild-type counterpart (Barra-Bily et al., 2010). Although the heat-shock type sigma factor RpoH2 is under control of σ^{EcfG} in this rhizobium, an *rpoH2* mutant strain is not heat sensitive (Oke et al., 2001; Ono et al., 2001). This observation rules out the involvement of RpoH2 in σ^{EcfG}-dependent heat resistance. Also, an *rpoE2* mutant was isolated in a screening for desiccation-sensitive mutants of *S. meliloti* (Humann et al., 2009; see Chapter 39), and *B. japonicum ecfG* and *phyR* mutants were found to be more desiccation sensitive than the wild-type strain (Gourion et al., 2009). Interestingly, trehalose is not only an osmoprotective compound, but is also known to protect bacteria against desiccation and heat. Thus, mutants of *R. etli*, *R. leguminosarum* bv. *trifolii* and *B. japonicum* deficient in trehalose synthesis were found to be not only osmosensitive but also heat and/or desiccation sensitive (McIntyre et al., 2007; Sugawara et al., 2010; Reina-Bueno et al., 2012). Therefore, although the σ^{EcfG}-dependent genes responsible for desiccation and heat resistance have not formally been identified, one can reasonably suspect trehalose synthesis genes to be involved in these processes.

Although some σ^{EcfG}-dependent genes could be associated with some σ^{EcfG} mutant phenotypes, a surprisingly large number of σ^{EcfG} targets encode proteins or sRNAs of unknown function. Interestingly, some of these proteins are conserved in the σ^{EcfG} regulons of other α-Proteobacteria, and are even part of general stress regulons of phylogenetically distant bacteria. For instance, the *S. meliloti* SMc00371 and *R. etli* CH00268 open-reading frames encode proteins homologous to the *E. coli* YciF, a σ^{S}-dependent gene of unknown function (Hindupur et al., 2006). Altogether, these findings suggest a universal role of these proteins in general stress responses, although their function is still unknown.

40.3.3.2 Symbiotic Phenotypes. Mutants of *S. meliloti rpoE2*, *R. etli rpoE4*, and *R. leguminosarum* bv. *viciae rpoZ* are able to form nodules with normal fixation efficiency on *Medicago sativa/truncatula*, *Phaseolus vulgaris*, and *Pisum sativum*, respectively (Wexler et al., 2001; Sauviac et al., 2007; Martínez-Salazar et al., 2009a; Humann et al., 2009). An explanation for this lack of symbiotic phenotype could reside in the functional redundancy of rhizobial genomes. For instance, *S. meliloti* RpoE2 controls the transcription of *katC* and *rpoH2*, but two additional catalases (KatA and KatB) and one additional heat shock-type sigma factor (RpoH1) are encoded by the genome. If no clear symbiotic phenotype could be associated with single *katC* or *rpoH2* mutations, *katA katC*, *katA katB*, and *rpoH1 rpoH2* double mutants displayed dramatically altered symbiotic phenotypes (Sigaud et al., 1999; Oke et al., 2001; Ono et al., 2001; Jamet et al., 2003; Mitsui et al., 2004). This suggests that the lack of some gene expression in the *rpoE2* mutant background could be compensated for by functionally redundant genes. Similar hypotheses can be made for *R. etli* (Martínez-Salazar et al., 2009b). In any case, the lack of symbiotic deficiency of the *ecfG* mutants cannot be explained by sigma factor inactivity *in planta*, since σ^{EcfG} target genes were found to be expressed in nodules induced by wild-type *S. meliloti* and *R. etli*, either by transcriptomic analyses (Capela et al., 2006) or through the use of transcriptional *lacZ* or *gus* fusions (Oke et al., 2001; Jamet et al., 2003; Martínez-Salazar et al., 2009a). Interestingly, the two *S. meliloti* genes examined using fusions (*katC* and *rpoH2*) revealed expression in specific zones of the *Medicago sativa* nodules (the infection zone and the late senescence zone), as well as in infection threads. No such expression pattern was detectable using the *katC–lacZ* fusion in an *rpoE2* mutant background, confirming that RpoE2 is indeed active in nodules (unpublished data). Whether *in planta* RpoE2 activation is a reflection of the numerous stress conditions encountered by bacteria during root infection and nodule senescence, or simply results from the nongrowing, stationary phase-like state of nodule bacteria, is currently unknown.

In contrast, *B. japonicum phyR* and *ecfG* mutants exhibited marked symbiotic phenotypes on soybean (*Glycine max*; two different cultivars) and mung bean (*Vigna radiata*) (Gourion et al., 2009). In all cases, nodule formation and development were delayed, the number of bacteroids was reduced, and nitrogen fixation was lowered in comparison to wild-type bacteria, with consequent impact on the general state of the plant (small size, chlorotic leaves). The color and morphology of the nodules were variable, ranging from normal red nodules to aberrant white nodules with signs of necrosis, and sometimes emerging ectopic root-like structures. Although the origin of this particular phenotype is so far unknown, it has been proposed to result from an imbalanced hormonal control of root versus nodule development, as a consequence of the inefficient bacterial colonization of the nodule (Gourion et al., 2009). Nevertheless, the reason of this poor colonization is not known, although we can reasonably assume that it results from the inability of the rhizobial mutants to cope with the stressful nodule environment.

40.4 CONCLUSIONS AND FUTURE PROSPECTS

It is now clear that σ^{EcfG} sigma factors are the long searched for regulators of the general stress responses in α-Proteobacteria. Although the main transduction cascade leading to σ^{EcfG} activation in response to stress is largely conserved among these bacteria, a number of fundamental issues remain unexplored. Notably, the numerous different conditions capable of inducing these responses, ranging from nutrient/energetic stresses to physico/chemical injuries, suggest the existence of various stress-sensing systems. However, in a given species, only a limited number of kinases/phosphatases have been identified on top of the σ^{EcfG} signal transduction cascades. This suggests that either of the additional systems integrate the signals upstream from the kinases, or that so far unidentified additional levels of regulation of the response exist. Studies in other bacteria such as *E. coli* have demonstrated that the general stress response can be regulated at multiple levels, including expression (transcription, RNA turnover, and translation), stability, and activity of the σ^{S} sigma factor. The finding that σ^{EcfG} activity or expression could be affected by (p)ppGpp or sRNAs is the first indication that σ^{EcfG} must also be viewed as potentially regulated at various levels. Moreover, the genes controlled by σ^{EcfG} sigma factors indicate that the response is structured as a complex network, including numerous additional regulators, like one and two-component systems, other sigma factors, and sRNAs, which can all integrate additional signals and regulate separate subsets of genes. The relative importance of all of these secondary levels of gene regulation needs to be considered in the future, as it can make the extent and nature of the response variable depending on the inducing cue. Finally, even though it is believed that the main function of the general stress response is to provide cells with a better stress resistance, the function of most of the genes regulated during this response is still unknown. Thus, possible impacts of the response on other aspects of cell physiology, for instance metabolism or bacterial envelope structure, must be the object of future studies. Other functions of the response in rhizobia may include adaptation to the specific environments they live in, such as soil and plant tissues.

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Section 8

Physiology and Regulation of Nodulation

Chapter 41

The Root Hair: A Single Cell Model for Systems Biology

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41.1 INTRODUCTION

As mentioned by Nelson et al. (2008), -omic studies performed on an entire tissue or organ composed of cells with different fates cannot provide a clear picture of the biological response of each cell type. This statement is especially true when referring to the initial steps of legume nodulation: the infection of the root hair cell, epidermal root cell type characterized by its polar elongation (Fig. 41.1), by nitrogen-fixing symbiotic bacteria. Applying a systems biology approach on a single differentiated cell type such as the root hair cell offers a unique opportunity to understand different aspects of plant cell biology. This chapter focuses on the strategies, challenges, and outcomes of a systems biology approach to study the response of the root hair cell, a model single cell type, to rhizobia inoculation.

41.2 THE ROOT HAIR SYMBIOTIC PATHWAY

Forward genetic approaches allowed the characterization of tens of legume genes controlling the infection process of the root hair cells. Mutants in these genes are defective in responding to the treatment of Nod factors, in root hair infection by rhizobia, and *a fortiori* in nodulation. Genetic analyses of these mutants led to the establishment of the symbiotic pathway (Fig. 41.2; see also Chapters 42, 43, 59, 110).

This pathway is initiated into the recognition of the nodulation factors (Nod factors), mixture of lipochito-oligosaccharides synthesized by the bacteria, by two-plant LysM receptor-like kinases. These receptors has been identified in *M. truncatula* (LysM receptor kinase 3 (LYK3) and Nod factor perception (NFP; see Chapter 51)) and in *L. japonicus* (Nod factor receptors 1 and 5 (NFR1, NFR5)). Thus, these receptors were identified based on the absence of root hair cell deformation and calcium spiking in response to rhizobia inoculation (Arrighi et al., 2006; Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003). For a long time, the interaction between the receptor kinases and the Nod factors was assumed, but only recently it was demonstrated (Broghammer et al., 2012).

Although both NFR1 and NFR5 contribute to Nod factor response, only NFR1 seems to have a functional kinase domain involved, among others, into the phosphorylation of NFR5 (Madsen et al., 2011).

Recent studies in *Medicago* support the idea of the reorganization of the localization of plasma membrane proteins including LYK3. This conclusion was reached after the observation of the colocalization of LYK3 with flotillin-like 4 (FLOT4), a membrane raft-associated protein-regulating root hair cell infection by rhizobia (Haney et al., 2011) and the interaction between LYK3 and the symbiotic remorin 1 (SYMREM1), another membrane raft-associated protein-controlling rhizobial infection (Lefebvre et al., 2010). The role of membrane raft during nodulation is

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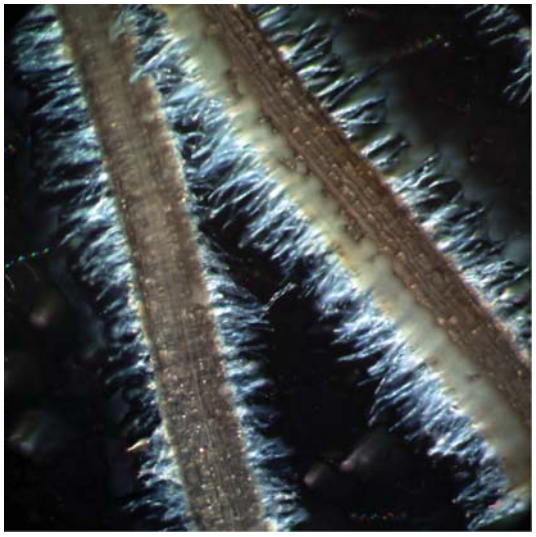

Figure 41.1 Root hair cells are single tubular root cells. Their distinctive lateral elongation increases the surface of exchange between the plant's root system and the soil. The main function of root hairs is to improve water and nutrient uptake by the roots. In legumes, they are the first site of infection by symbiotic nitrogen-fixing bacteria.

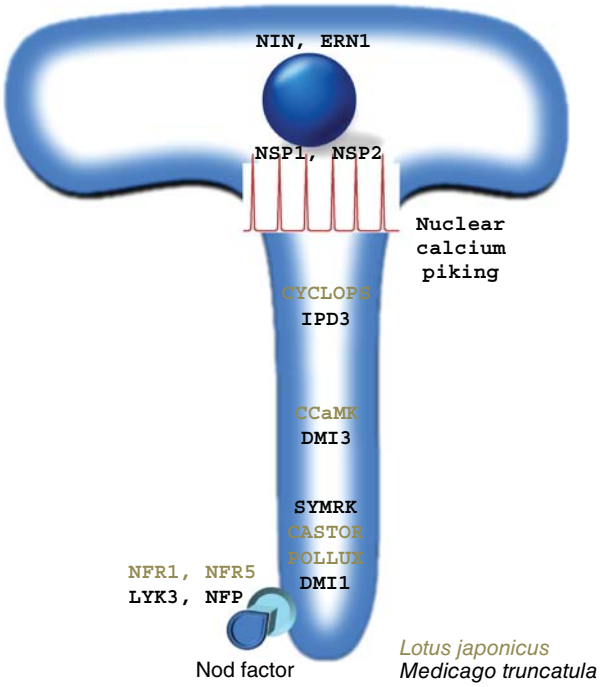

Figure 41.2 Signaling cascade activated in response to the recognition of the symbiotic nitrogen-fixing bacteria. This cascade is leading to the activation of a set of TFs (red circles), which is necessary to a successful infection of the root hair cells by the bacteria.

also supported by the essential function of FLOT2 during rhizobial infection of the root hair cells (Haney and Long, 2010).

Additional plasma membrane proteins are required to transduce the Nod factor signal in root hair cell. The *L. japonicus SYMbiosis Receptor-like Kinase* (*SYMRK*) and the *M. truncatula Does not Make Infections* (*DMI2*) genes act early in the signaling cascade, potentially directly under the control of the receptor lysine kinases (Endre et al., 2002; Stracke et al., 2002). The molecular function of these plasma membrane proteins is not currently characterized.

One well-characterized response of the root hair cells to Nod factor treatment or rhizobia inoculation is the oscillation of calcium concentration in the nucleoplasm and perinuclear cytoplasm (Capoen et al., 2011; see Chapters 54, 57). To capture these oscillations, researchers used fluorescent proteins or dyes sensitive to calcium concentrations such as the YC2.1 cameleon protein (Capoen et al., 2011) and the Oregon Green dextran (Imaizumi-Anraku et al., 2005).

By expressing the cameleon protein in roots, Kosuta et al. (2008) analyzed the amplitude and frequency of the oscillations of calcium of *M. truncatula* in response to *Sinorhizobium meliloti* and *Glomus intraradices* inoculations, the symbiotic bacterium, and fungus involved in nodulation and mycorrhization, respectively. Interestingly, Kosuta et al. (2008) observed different amplitudes and frequencies of calcium spikes depending on the nature of the microbes. This result was not confirmed by Sieberer et al. (2012; see Chapter 57). Nevertheless, it is unclear whether the frequency and the amplitude of the calcium oscillation is utilized by the plant cell to distinguish between rhizobia and mycorrhiza infection. If this is the case, it is attempting to hypothesize that the nature of the calcium oscillations would control the expression of a specific set of genes required for mycorrhization or nodulation, as previously reported by other groups using different systems (Song et al., 2012).

To date, the relationship existing between the recognition of the Nod factor by LysM receptor lysine kinases and their phosphorylation with the activation of calcium oscillations has not been revealed. One hypothesis would be the production of an isolated and related secondary messenger in response to the recognition of the Nod factor, such as the products of the degradation of phospholipids by the phospholipase C and phospholipase D (Charron et al., 2004; den Hartog et al., 2001; Engstrom et al., 2002; Pislariu and Dickstein, 2007). Another potential secondary messenger is mevalonate. Kevei et al. (2007) highlighted the interactions between *M. truncatula* DMI2 with 3-hydroxy-3-methylglutaryl CoA reductase 1 (HMGR1), a key enzyme in the biosynthesis of mevalonate, and controlling nodulation. However, the direct effect of these putative secondary messengers on calcium oscillation has not been demonstrated.

In *L. japonicus* and *M. truncatula*, calcium oscillations are under the control of the nuclear membrane cation

channels LjCASTOR, LjPOLLUX, and MtDMI1 (Ane et al., 2004; Capoen et al., 2011; Charpentier et al., 2008; Imaizumi-Anraku et al., 2005; Riely et al., 2007). To balance the influx and efflux of calcium in the nucleus, MCA8 encoding a calcium ATPase acts as a calcium pump responsible for the movement of calcium back to the nuclear membrane (Capoen et al., 2011). Calcium oscillations are perceived by the nuclear protein calcium- and calmodulin-dependent serine/threonine protein kinase (CCaMK in *L. japonicus*; DMI3 in *M. truncatula*) (Levy et al., 2004; Mitra et al., 2004). CCaMK has an integrative role in the Nod factor-associated signaling cascade since its only activation is sufficient to induce nodulation even in absence of rhizobia (Gleason et al., 2006; Tirichine et al., 2006; see Chapter 54). The binding of calcium ions to the calmodulin and EF-hand motifs of CCaMK protein induce the autophosphorylation of the kinase domain of the protein. The *L. japonicus* CYCLOPS and *M. truncatula* IPD3 proteins are phosphorylated by CCaMK (Horvath et al., 2011; Messinese et al., 2007; Ovchinnikova et al., 2011; Yano et al., 2008). The role of these nuclear calcium oscillations and the function CCaMK protein has not been resolved, but it is likely possible that they regulate the transcriptional activity of transcription factor acting downstream in the symbiotic pathway.

Transcription factors are master regulators of root hair cell response to rhizobia inoculation including the *Nodulation Signaling Pathway 1 and 2* (*NSP1* and *NSP2*) genes encoding GRAS transcription factors (Kalo et al., 2005; Smit et al., 2005). *In vivo* and *in vitro* experiments highlighted the direct interactions between NSP1 and NSP2 (Hirsch et al., 2009). In the same study, Hirsch et al. (2009) also demonstrated the binding of NSP1 to *cis*-elements located in the promoter region of the *Early Nodulin 11* (*ENOD11*) gene, which transcriptionally induced in response to rhizobia inoculation (Journet et al., 2001). Additional interactions between legume transcription factors and the promoter sequence of genes known to control nodulation has been reported. For example, using the yeast one-hybrid method, Andriankaja et al. (2007) demonstrated the binding of Ethylene Response Factor Required for Nodulation1 [ERN1], transcription factor acting early during the nodulation process (Middleton et al., 2007), with the *Medicago* ENOD11 promoter. More recently, Soyano et al. (2013) characterized the direct regulation of the expression of *L. japonicus NF-YA1* and *NF-YB1*, two activators of cortical cell division, by the NIN transcription factor encoding an RWP-RK transcription factor (Marsh et al., 2007).

The molecular mechanisms controlling the activity of these transcription factors in root hair cells are unknown. To date, the soybean orthologs of these transcription factors are upregulated in root hair cell in response to *B. japonicum* (Libault et al., 2010a).

41.3 SYSTEMS BIOLOGY APPROACH TO ELUCIDATE THE MOLECULAR RESPONSE OF ROOT HAIR CELLS IN RESPONSE TO RHIZOBIA

Our current knowledge of the genetic pathway controlling root hair cell response and infection by rhizobia is limited to several genes. There is no doubt that these genes are master regulators of the root hair cell infection based on the drastic defect in nodulation observed in related mutants. However, due to the complexity of the root hair response to rhizobia (i.e., recognition of the symbiont, deformation, and infection of the root hair cell), it is expected that a higher number of genes contribute to regulate the various aspects of the root hair cell response to rhizobia. These genes are not characterized, yet potentially due to functional redundancy between homologs or due to their subtle function in regulating legume root hair cell infection and nodulation. Reverse genetic and systems biology approaches represent a potential solution to identify these genes.

To specifically access a root hair cell before analyzing its biology and response to rhizobia in detail, researchers took advantage of the characteristic polar growth of the root hair cells (Fig. 41.1) to isolate them from the rest of the root system. The methodologies used to reach this goal vary according to the plants used and, more specifically, the nature of the root system. Nevertheless, these methods are based on the breaking of the thin root hair cells into liquid nitrogen followed by filtration steps to separate root hairs and the remaining root system. The methods currently used are as follows: (1) brushing of the root system in liquid nitrogen (Ramos and Bisseling, 2003); (2) gentle stirring of the root system in liquid nitrogen (Lauter et al., 1996). The first method is time consuming but more adequate to isolate root hair cell from fragile root system such as *M. truncatula*; whereas the second method allows a higher yield in root hair cells in a minimum time but is more destructive. As a consequence, contamination of the root hair cell preparation by fragmented roots is a major limitation. The filtration of the root hair cell preparation enhances the purity of the root hair cell preparations.

Among model legumes, root hair cells from *Medicago* and soybean have been isolated and used to analyze the response of the cell type to rhizobia inoculation. In *Medicago*, the transcriptomic response of the root hair cells to *S. meliloti* inoculation is currently being investigated [Dr. Jeremy Murray, John Innes Center, personal communication]. Since 2005, soybean has been extensively used to investigate root hair cell response to rhizobia inoculation owing to the ease to isolate gram quantities of root hair cells (i.e., as much as 1 g of root hair cells is isolated from 1000 seedlings). The application of various -omic methods

including transcriptomics (Libault et al., 2010a), proteomics (Brechenmacher et al., 2009; Brechenmacher et al., 2012), metabolomics (Brechenmacher et al., 2010), and phosphoproteomic analyses (Nguyen et al., 2012) on these soybean root hair cell preparations led to an impressive collection of data, clearly highlighting the complexity of the response of the root hair cells to rhizobia. These data were collected over time to monitor the dynamic changes of the root hair cell response to rhizobia at each stage of the infection process.

41.4 TRANSCRIPTOMIC APPROACH TO UNRAVEL THE COMPLEXITY OF THE ROOT HAIR CELL RESPONSE TO RHIZOBIA

To broadly analyze the transcriptional response of the root hair cells to rhizobia, both Affymetrix microarray and Illumina RNA-seq technologies were used. These technologies led to the identification of 1973 soybean genes differentially regulated in root hair cells in the few hours following *B. japonicum* inoculation (e.g., 12, 24, and 48 h after bacterial inoculation (Libault et al., 2010a)). Among these genes, the soybean gene orthologs to the receptor lysine kinase *NFR1* (*LYK3*), *NFR5* (*NFP*) were clearly upregulated in response to bacteria inoculation. In addition, among the 13 soybean genes orthologous to *M. truncatula* and *L. japonicus NSP1*, *NSP2*, *NIN*, and *ERN1*, five clearly show an induction of their expression in root hair in response to *B. japonicum* inoculation (Fig. 41.3).

Including these genes, a total of 233 genes were consistently regulated during soybean root hair infection. Looking at the identity of the 233 genes differentially expressed in soybean root hair cells, a significant percentage of them control cell wall biosynthesis and degradation (12 genes), the plant defense system (21 genes), and regulate transcriptional activity (19 genes; Fig. 41.4).

It is tempting to hypothesize that these 19 *B. japonicum*-inducible root hair transcription factor genes control the broad transcriptomic regulation of the root hair transcriptome in response to rhizobia. Ideally, the target genes of these transcription factors should be characterized specifically in root hair cells before and after the inoculation by rhizobia to enhance our knowledge of the symbiotic pathway. In addition, we cannot omit the fact that these transcription factors are responsible of the upregulation and then downregulation of the expression of defense-related genes as previously reported (Libault et al., 2010a). This is an essential aspect of legume nodulation because the finality of the nodulation is to allow the infection of plant cells by the symbiotic bacteria. The third category of genes strongly regulated upon *B. japonicum* inoculation is involved in

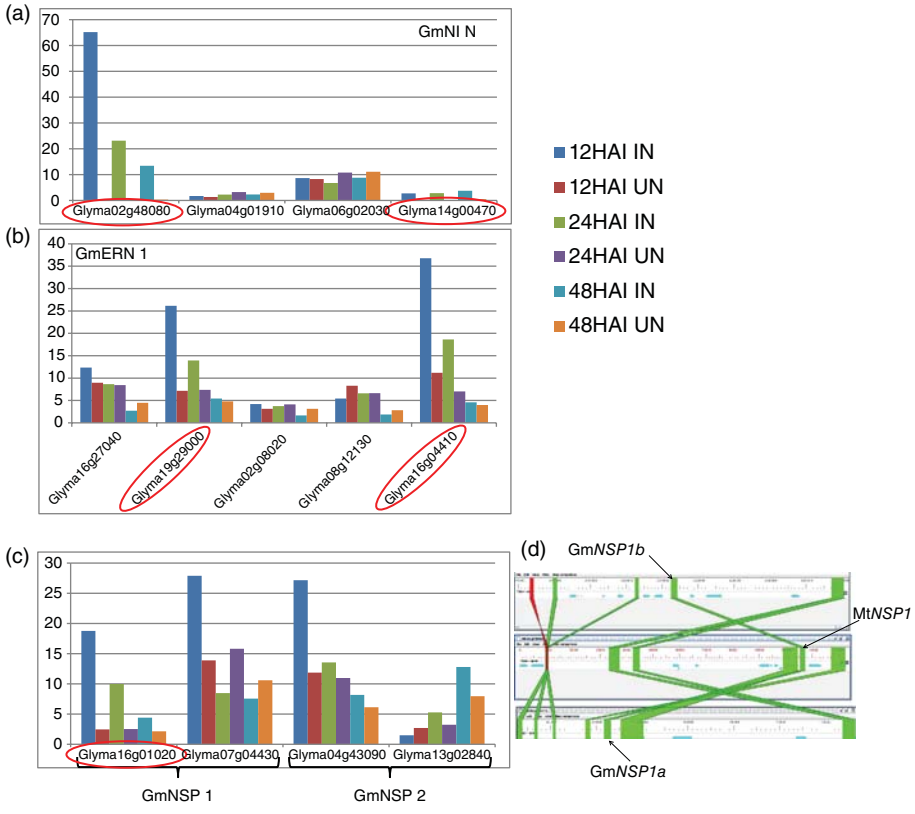

Figure 41.3 Relative expression levels of soybean genes orthologous to *Medicago* and lotus NIN (a), ERN1(b), *NSP1*, and *NSP2* (b) during the early stages of nodulation. Normalized gene expression (*y*-axis) was quantified using RNA-seq technology in root hair cells inoculated (IN) and mock-inoculated (UN) by *B. japonicum* and at three different times of the infection (i.e., 12, 24, and 48 h after inoculation (HAI)). Differentially expressed genes are highlighted in red. (d) Microsynteny relationships between MtNSP1 gene located on chromosome 8 and two soybean orthologs, GmNSP1a (Glyma16g01020) and 1b (Glyma07g04430), respectively. Synteny relationships between legume genes are highlighted in green and red blocks (the latter reflects inversions of the direction between orthologs).

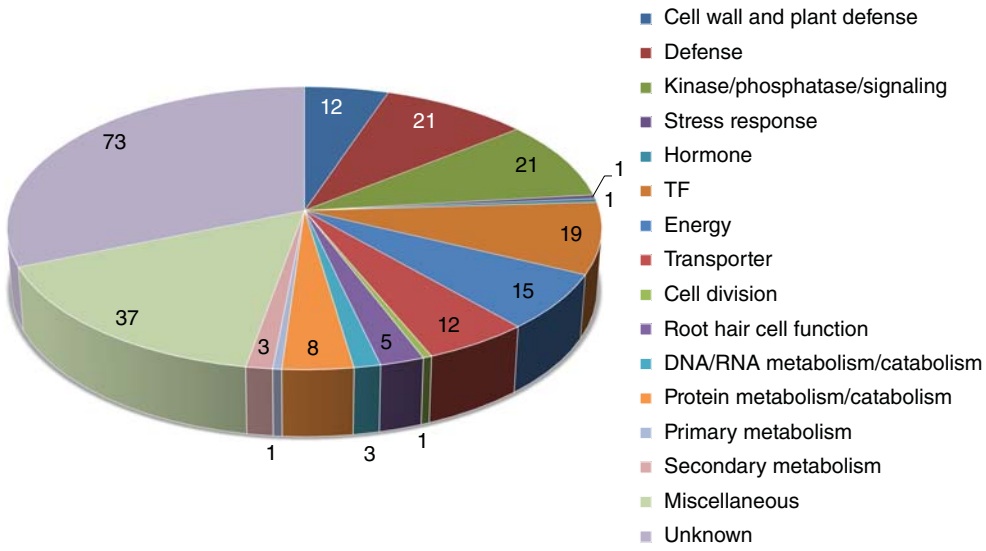

Figure 41.4 Distribution in functional categories of the soybean genes repetitively transcriptionally regulated 12, 24, and 48 h after *B. japonicum* inoculation (Libault et al., 2010a).

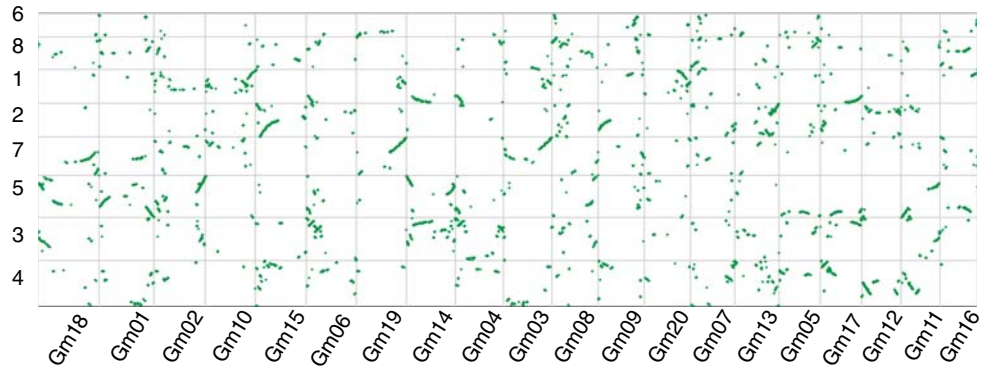

Figure 41.5 Macrosynteny relationships between the eight *Medicago* chromosomes (*y*-axis) and the 20 soybean chromosomes (*x*-axis). Each green dot on the graphic reveals synteny relationships between the two legumes. This figure was generated by the CoGe system (http://genomevolution.org/CoGe/).

cell wall biosynthesis and degradation. Many studies in *Arabidopsis* clearly connected changes in the composition of root hair cell wall with calcium oscillations and modifications in the extracellular pH and accumulation of reactive oxygen species as major regulators of root hair cell elongation (Monshausen et al., 2007; Monshausen et al., 2009; Monshausen et al., 2008). In legumes, the impact of reactive oxygen species on root hair cell elongation and deformation has been reported in response to the treatment of Nod factors or rhizobia inoculation (Cardenas et al., 2008; Lohar et al., 2007). Hence, it seems plausible that at least a subset of the genes and proteins controlling root hair cell elongation had been neofunctionalized to control root hair curling during the early stages of nodulation.

These 233 genes could be considered as valuable candidate for functional genomic studies based on their probable role during soybean root hair infection process. Using more stringent criteria, 41 soybean genes should receive careful attention from the legume community, considering the specific activation of their transcription only upon rhizobia inoculation (i.e., no transcripts were detected in various tissues, and, more interestingly, in mock-inoculated root hairs). Taking advantage of the strong synteny between the *Medicago* and soybean genome (Fig. 41.5) and the upcoming availability of the *Medicago* root hair transcriptome, our understanding of the common mechanisms of root hair cell infection by rhizobia between determinate and indeterminate nodulations will be revealed.

41.5 CHARACTERIZATION OF THE SOYBEAN ROOT HAIR PROTEOME, PHOSPHOPROTEOME, AND METABOLOME

To complement the root hair transcriptome and because the goal of the root hair systems biology approach is to provide an integrated and meaningful understanding of root hair cell infection by rhizobia, the root hair cell proteome has been characterized as well as its perturbation in response to rhizobia. The large quantities of soybean root hair cells are compatible with the sequencing of a large population of peptides, leading to an impressive collection of root hair cell proteins and the establishment of the first root hair proteome map. The soybean root hair protein map is the result of two complementary studies. The first study was published in 2009 combining 2D gel electrophoresis and 1D PAGE-liquid chromatography with multidimensional protein identification (MudPIT) technology (Brechenmacher et al., 2009). This study led to the identification of 1492 root hair cell proteins and clearly highlighted the complementarity of the different proteomic methods used to identify them. The functional categorization of these proteins clearly support the basic function of the root hair cell (i.e., many water and ion transporters and protein controlling root hair cell elongation had been identified). This first analysis was updated in 2012 using accurate mass and time (AMT) tag method (Brechenmacher et al., 2012). Owing to the higher sensitivity of the method used, the updated soybean root hair proteome map now encompasses 8262 proteins. As expected, the AMT approach identified 94% of the protein previously identified by 2D-gel SDS-PAGE and MudPIT approaches.

The differential abundance of root hair protein in response to rhizobia has been initiated using the soybean root hair as model. A total of 26 root hair proteins were differentially abundant in response to *B. japonicum* using a 2D SDS-PAGE approach (Wan et al., 2005). Among them, the abundance of a soybean phospholipase D significantly increases 6 h after *B. japonicum* inoculation. As mentioned earlier, this protein might play a critical role in the production of secondary messenger in the symbiotic pathway (den Hartog et al., 2001).

The functional characterization of the root hair cell proteins found in different abundance in response to *B. japonicum* is essential to a systems biology understanding of the infection of this single cell type.

To complement these investigations and based on the importance of posttranslational modification of proteins in the symbiotic pathway, the dynamic changes of the soybean root hair phosphoproteome in response to rhizobia have been investigated. To do so, Nguyen et al. (2012) digested root hair proteins inoculated and mock-inoculated with *B. japonicum* with trypsin; enriched their preparation in phosphopeptide using the nickel–nitriloacetic acid enrichment method; and then identified the phosphopeptides using reversed-phase liquid chromatography-tandem mass spectrometry. Not surprisingly, most of phosphorylation changes occurred within the first hours after inoculation. This very early response might be a reflection of the predominant role of the receptor kinases in the symbiotic pathway rapidly activated in response to rhizobia. Among the phosphoproteins identified, the phosphorylation level of 240 root hair cell proteins was significantly changed in response to *B. japonicum* inoculation including protein-controlling chromatin condensation, transcription, and translation. Combined with the transcriptomic study (Libault et al., 2010a), this result supports an early response of the root hair cell to rhizobia inoculation under the form of a massive posttranslational change of proteins (with a maximum at 3 h after inoculation), likely initiated by the receptor lysine kinases, and then a late response in the nuclei where nodulation-related transcription factors (e.g., NSP1, NSP2, and ERN1) will regulate the expression of hundreds of genes required to initiate the infection process with a maximum response between 12 and 48 h after inoculation.

To complement proteomic and phosphoproteomic studies, the root hair cell metabolome and its regulation in response to *B. japonicum* has been characterized. Among the 2610 root hair cell metabolites detected, 166 were found in different abundance in response to *B. japonicum* inoculation. Interestingly, flavonoids, plant metabolites activating the transcription of the Nod genes in bacteria, were found in higher abundance in inoculated root hair cell supporting the idea of a positive loop to enhance plant–bacteria interaction. Also, carbohydrates were also strongly accumulated in root hair cells in response to *B. japonicum* including trehalose. The use of *B. japonicum* mutants defective in trehalose biosynthesis supporting the root hair trehalose has a bacterial and not a plant origin. Because trehalose is an osmoprotectant, this accumulation of trehalose in infecting *B. japonicum* suggests the perception of an osmotic stress during the infection process by the bacteria.

41.6 UPCOMING CHALLENGES IN ROOT HAIR CELL SYSTEMS BIOLOGY

A systems biology analysis of root hair cell infection by rhizobia requires interdisciplinary studies. Hence, a major challenge in systems biology is the meaningful integration of various data sets (i.e., genomic, transcriptomic, proteomic, and metabolomic), leading to a deep understanding of the response of the root hair cell to rhizobia. The ease to isolate root hair cells and the development of methods to globally analyze their response to rhizobia inoculation are clear advantages to successfully apply a systems biology approach

because the data sets generated will not suffer from the multicellular complexity of plant organs and, as a consequence, will be unambiguous.

To enhance our understanding of the root hair cell infection, in addition to existing resources, additional aspect of root hair cell biology must be studies such as the compartmentalization of proteins and metabolites in root hair cell organelles (e.g., membrane rafts, liquid-ordered microdomains in cell membranes, are playing an important role during nodulation (Lefebvre et al., 2010)). A second field of investigation would be the characterization of the root hair epigenome and its regulation in response to rhizobia inoculation.

Ultimately, systems biology will unravel the positive and negative regulations of gene regulatory, proteomic, and metabolic pathways in root hair cells in response to rhizobia. Working at the level of one single cell type will help to generate and test computational model than to validate these networks. However, preliminary analyses clearly highlighted that gene expression and protein abundance are not tightly correlated even when working at the level of one single cell type such as the root hair cell (Libault et al., 2010b).

To analyze and integrate the various data sets, bioinformatic tools are needed. Many bioinformatics resources are currently available to visualize and to compare genomic, transcriptomic, proteomic, and metabolomics data sets such as the Legume Information System (http://www.comparative-legumes.org), Phytozome (http://www.phytozome.net/), SoyBASE (Grant et al., 2010), and SoyKB (Joshi et al., 2012). More recently, the iRootHair database has been created allowing researchers to access root hair resources even from nonlegume plants (Kwasniewski et al., 2013). Altogether, the use of these tools, the development of new computational resources, and the production of reliable data sets will help to unravel the root hair response to rhizobia inoculation.

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Chapter 42

How Transcriptomics Revealed New Information on Actinorhizal Symbioses Establishment and Evolution

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42.1 INTRODUCTION

Two root nodule symbioses are known between nitrogen-fixing soil bacteria and higher plants: legumes (as well as the nonlegume *Parasponia*) associated with Rhizobia and plants collectively designated actinorhizal that interact with *Frankia*. In both cases, the microsymbionts induce the formation of special organs on the roots of their host plants, named root nodules able to fix nitrogen. Legume and actinorhizal nodules differ in their ontogeny and structure (Franche et al., 1998; Wall, 2000, Pawlowski, 2009; Santi et al., 2013). Both legumes and actinorhizal plants belong to the Rosid I clade, thus suggesting that they share a predisposition for symbiosis (Soltis et al., 1995; Doyle, 1998,

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2011; Bell et al., 2010). Several independent evolutionary origins have been postulated for rhizobial symbioses (Doyle, 2011) and three to four for actinorhizal symbioses (Swensen, 1996). The molecular bases of this predisposition are not yet known.

Molecular approaches have progressed for actinorhizal species during last decades mainly for the temperate species, *Alnus glutinosa* and *Datisca glomerata*, and the tropical trees of the Casuarinaceae family (Obertello et al., 2003; Laplaze et al., 2008; Bogusz and Franche, 2012, Svistoonoff et al., 2014). For the latter, transgenic plants have been obtained (Franche et al., 1998), and recent work shows the successful application of a RNA silencing system (RNAi) using hairy root in *Casuarina glauca* and in *Datisca glomerata* (Gherbi et al., 2008; Markmann et al., 2008). Thus, actinorhizal symbioses emerged recently as simple and original systems of root nodule initiation, and they offer the opportunity to explore developmental strategies significantly different from legumes to form nitrogen-fixing nodules.

One of the key goals for understanding actinorhizal root nodule symbiosis is the identification of the network of plant and bacterial determinants whose gene products interact to initiate root nodule formation and to support the nitrogen-fixation process. Recent years have witnessed intense growth of different genomic approaches (ESTs, microarray, and deep sequencing) for high-throughput analyses of genes and their transcripts. This was recently applied to different actinorhizal systems, and we review here the current state of knowledge on the genomics of the actinorhizal symbiotic interaction.

42.1.1 Actinorhizal Species

Actinorhizal plants represent about 200 species encompassing 25 genera in eight different angiosperm families, in three different orders (Huss-Danell, 1997): the Betulaceae, Casuarinaceae, and Myricaceae of the order Fagales; the Rosaceae, Rhamnaceae, and Elaeagnaceae of the order Rosales; and the Coriariaceae and Datiscaceae of the order Cucurbitales (Vessey et al., 2004). In some families all members are nodulated (Coriariaceae, Elaeagnaceae, Datiscaceae, and Casuarinaceae), whereas in others, only a portion of the genera are nodulated (Betulaceae, Myricaceae, Rhamnaceae, and the Rosaceae). They are widely distributed, found on all continents except in Antartica, are perennial dicotyledonous angiosperms, and are, with the exception of the genera *Datica*, woody trees or shrubs. Examples of well-known genera include *Alnus* (alder), *Elaeagnus* (autumn olive), *Hippophae* (sea buckthorn), and *Casuarina* (she oak). Most actinorhizal plants are capable of high rates of nitrogen fixation comparable to those found in legumes (Torrey, 1976). Ecologically, actinorhizal plants are usually pioneers on nitrogen-poor soils, and are frequently found in relatively harsh sites, including glacial till, new volcanic soil, sand dunes, clear cuts, and desert and chaparral (Schwencke and Caru, 2001). As a consequence, these plants play an essential role in land stabilization and soil reclamation (Sprent and Parsons, 2000). Actinorhizal plants have been used in erosion control, soil reclamation, agroforestry, and dune stabilization, as well as in fuel production. The properties of actinorhizal plants as pioneer species are linked mainly with the ability of their root to develop original organs, the nodules, containing the symbiotic N-fixing bacteria, in response to low nitrogen conditions.

42.1.2 The Actinomycete *Frankia*

The microsymbiont *Frankia* is a filamentous Gram-positive actinomycete and is characterized by a slow growth rate and high G + C DNA content (Simonet et al., 1990; Benson and Silvester, 1993). In pure culture, *Frankia* presents three major structures: vegetative hyphae (multiplication form), vesicles that are the site of nitrogen fixation, and sporangia (dissemination form). No *Frankia* strain specific to a single host plant species has been described to date (Pawlowski and Sprent, 2008). Nevertheless, host specificity is present at different levels and a broad correspondence can be defined between the phylogenies of *Frankia* strains and actinorhizal plants (see Chapter 24, this book).

Due to the lack of genetic tools (Mullin and Dobritsa, 1996), most aspects of *Frankia* biology, particularly symbiosis, are still unknown (Perrine-Walker et al., 2011). Several trials of genetic transformation, mutagenesis, and functional complementation failed to provide conclusive results. The recent sequencing of different *Frankia* genomes (Normand et al., 2007a,b; Persson et al., 2011) should bring new information. This is detailed in Chapter 24.

42.1.3 Development of Actinorhizal Nodules

Actinorhizal nodule development only occurs under conditions of nitrogen deprivation, and it is assumed that plant root emit signals of still unknown nature that are perceived by *Frankia*. Signal exchange between *Frankia* and the host plant has been investigated by several laboratories (Prin and Rougier, 1987; van Ghelue et al., 1997; Ceremonie et al., 1999). Root flavonoids are prime candidates for plant signaling molecules, since they govern plant–microorganism cross talk in the rhizosphere and regulate the expression of *nod* genes in *Rhizobium*, prompting thus synthesis of the rhizobial signal molecule, the so-called nodulation (Nod) factors (Abdel-Lateif et al., 2012; Chapters 50, 51).

On the bacterial side, a partial purification of a root hair deforming factor from the supernatant of *Frankia alni* cultures was achieved using root hair deformation assay on *Alnus*. This *Frankia* root hair deforming factor was

found to be of different nature than Nod factors (Ceremonie et al., 1999). Complete genome sequencing of *Frankia* strains infective on Alnus (ACN14) and Casuarina (CcI3) confirmed so far the absence of the canonical Rhizobia *nodABC* genes (Normand et al., 2007a; Hocher et al., 2012) in these strains. However, the recent sequencing of the strain *Candidatus Frankia datiscae* could bring new insights into the nature of the *Frankia* molecule signal (Persson et al., 2011). Several approaches are presently underway to isolate and characterize further the root hair deforming factor.

Depending on the host plant, two modes of infection of actinorhizal plants by *Frankia* have been described: intercellular root invasion and intracellular root hair infection (Berry and Sunnel, 1990; Duhoux et al., 1996; Wall and Berry, 2008). Intracellular infection (e.g., of *Casuarina, Alnus,* and *Myrica*) starts with root hair curling induced by an unknown *Frankia* signal. *Frankia* penetrates the curled root hairs and infection proceeds intracellularly in the root cortex (Lalonde and Knowles, 1975; Berg, 1990). At the same time, limited cell divisions occur in the cortex, leading to the formation of a small external protuberance called the prenodule (Berry and Sunnel, 1990). Most of prenodule cells are infected with *Frankia*. But, while cortical cell divisions lead to the formation of a nodule primordium in legumes, actinorhizal prenodules do not evolve in nodules. The function of the *C. glauca* prenodule is not yet fully understood, but studies of the expression of symbiosis-related genes (*cg12*, *cghb*, see above for details) coupled to cellular modification (cell wall lignification) indicated that the prenodule displays the same characteristics as the nodules and can be considered as a very simple symbiotic organ (Laplaze et al., 2008; see also Chapter 55).

The prenodule could thus be a parallel symbiotic organ on its own or the remaining form of a common nodule ancestor for legumes and actinorhizal plants (Gualtieri and Bisseling, 2000; Laplaze et al., 2008).

Concomitant with prenodule development, cell divisions are induced in pericycle cells opposite to a protoxylem pole, giving rise to an actinorhizal lobe primordium. Concerning the intercellular root invasion pathway (e.g., *Discaria*, *Ceanothus*, *Elaeagnus*, and *Hypophae*), *Frankia* hyphae penetrate between two adjacent rhizoderm cells and progress apoplastically through cortical cells within an electron-dense matrix secreted into the intercellular spaces (Miller and Baker, 1985; Racette and Torrey, 1989; Liu and Berry, 1991; Valverde and Wall, 1999). Unlike the intracellular mode of infection, no prenodule is formed in the root cortex. Once the nodule primordium has developed from the pericycle, intracellular penetration by *Frankia* and the formation of infection threads is initiated acropetally in developing cortical cells of the nodule lobe primordium, following a pattern similar to that described in plant species invaded through root hairs (see also Chapter 55).

The mature actinorhizal nodule consists of multiple lobes, each of which is a modified lateral root. In each lobe there is a central vascular bundle, and *Frankia* is restricted to the cortical cells. Four zones were characterized in actinorhizal nodules: the meristem zone (I) at the apex of nodule, this zone is responsible for indeterminate growth of nodule and always free from *Frankia;* the infection zone (II) is adjacent to the apical meristem, the hyphae infect some of the new cells derived from meristem activity that subsequently enlarge; and the fixation zone (III) is composed of infected and uninfected cells. Within this zone, infected host cells are hypertrophied. *Frankia* hyphae and vesicles are present, *Frankia nif* genes, coding for the nitrogenase complex, are expressed (Pawlowski et al., 1995), and the nitrogenase protein is detected (Huss-Dannell and Bergman, 1990). Therefore, in this zone, active nitrogen fixation takes place and the uninfected cells are smaller than infected cells; the senescence zone (IV) in older nodule lobes where host cytoplasm and endophyte degeneration is observed (Newcomb and Wood, 1987), *nifH* expression is switched off, and nitrogenase activity is lost in legume and actinorhizal nodules (Vikman et al., 1990; Swaraj et al., 1993).

42.1.4 Molecular Events Associated with Nodulation in Actinorhizal Species

During differentiation of the symbiotic actinorhizal root nodule, a set of genes – called actinorhizal nodulin genes – is activated in the developing nodules (Mullin and Dobritsa, 1996, Pawlowski et al., 1997). Similar to legumes, two major types of actinorhizal nodulin genes have been defined by their pattern of expression and function. Early nodulin genes are expressed before the beginning of nitrogen fixation; they are thought to be involved in plant infection or in nodule organogenesis, whereas late nodulin genes comprise sequences involved in different metabolic activities necessary for the functioning of the nodule (Obertello et al., 2003). Many nodule-specific or nodule-enhanced expressed genes have been detected in several actinorhizal plants including *Alnus*, *Datisca*, *Elaeagnus*, and *Casuarina* and were reviewed by several authors (Pawlowski and Bisseling, 1996; Wall, 2000; Hocher et al., 2006; Laplaze et al., 2008, Chapter 43, this book).

42.1.5 Genomic Resources for Actinorhizal Plant Species

Our group in collaboration with P. Normand's group has developed the first genomic platform to identify plant genes involved in the symbiotic process between *Frankia* and actinorhizal species (Hocher et al., 2006, 2011a). Starting from cDNA libraries obtained from noninoculated roots (controls), inoculated roots (2, 4, and 7 days post-inoculation

(dpi)) and nodules (3 weeks post-inoculation), two sets of ESTs were sequenced for *Casuarina glauca* and *Alnus glutinosa*. For both species, the ESTs database contains a total of about 35 000 valid ESTs. Each set of ESTs sequence data was processed and annotated using a multimodule custom pipeline as described in Hocher et al., (2006, 2011a,b) to generate a set of nonredundant gene sequences. The clustering resulted in about 15 000 unigenes for *A. glutinosa* and for *C. glauca.* To analyze transcriptional changes occurring during symbiosis in *A. glutinosa* and *C. glauca*, a 15K Agilent custom oligonucleotide chip was designed for each species. Two biological conditions were compared for both plants: noninoculated roots and 3-week-old nodules and resulted in the identification of sets of differentially expressed genes for *C. glauca* and *A. glutinosa* (Hocher et al., 2011a).

42.1.6 Genetic Program Recruited During Symbiosis of Actinorhizal Species

The global analysis of our data revealed that 11 to 14% of the 15 000 unigenes were regulated or specifically induced during nodule development for *A. glutinosa* and *C. glauca*, respectively. A majority of them are linked to transport, metabolism, protein synthesis machinery, cell wall, defense, and response to stress indicating that nodulation is associated with a high metabolic activity. This situation was found to be very close to that described in different transcriptomic studies performed in model legumes (Asamizu et al., 2000; Journet et al., 2002; Colebatch et al., 2004; El Yahyaoui et al., 2004; Maunoury et al., 2010; see also Chapter 41). For the first time, a similar global behavior was found during nodulation of two actinorhizal species and this behavior was found very similar to what is described for legumes, thus suggesting similar genetic programs for root nodule symbioses.

Furthermore, among the regulated genes, all the different nodulins already described in *A. glutinosa* and/or *C. glauca* (Goetting-Minesky and Mullin, 1994; Ribeiro et al., 1995, 1996; Guan et al., 1996a,b, 1997; van Ghelue et al., 1996; Pawlowski et al., 1997, 2003; Jeong et al., 2004; Svistoonoff et al., 2003) were identified in both species with a similar regulation pattern (Hocher et al., 2011a).

42.1.7 Role of Flavonoids in Actinorhizal Symbiosis

If several studies have reported the implication of flavonoids during actinorhizal plants–*Frankia* interaction, direct evidence of flavonoids as early signals between plant and *Frankia* is still lacking (Perrine-Walker et al., 2011; Abdel-Lateif et al., 2012). *Casuarina glauca* transcriptome analysis brought new insights into a putative role of flavonoids during actinorhizal symbiosis. Eight genes implicated in a flavonoid biosynthetic pathway were identified and a kinetic study of their expression during a nodulation time-course revealed a higher accumulation of isoflavone reductase transcripts as soon as 12 h after inoculation of the plant by the bacteria (Hocher et al., 2006; Auguy et al., 2011). These data suggest that flavonoids could act as plant signals in actinorhizal symbioses and might influence the symbiotic specificity in actinorhizal symbioses (Hocher et al., 2011b). However, direct evidence of their involvement is still lacking. We thus used RNA interference to silence chalcone synthase, the enzyme involved in the first committed step of the flavonoid biosynthetic pathway, in *Casuarina glauca*. Plants impaired in flavonoid biosynthesis were generated and used to study flavonoid accumulation and further nodulation. Knockdown of chalcone synthase expression reduced the level of specific flavonoids and resulted in severely reduced nodulation. Nodule formation was thus rescued by supplementing the RNAi plants with naringenin, an upstream intermediate in flavonoid biosynthesis. For the first time, a direct evidence of an essential role for flavonoids during the early stages of actinorhizal nodulation was demonstrated (Abdel-Lateif et al., 2013).

42.1.8 An Actinorhizal Signaling Pathway

In legumes, recent work using model species as *Medicago truncatula* and *Lotus japonicus* elucidated the role of many genes essential for the different nodulation steps (Oldroyd et al., 2009; Oldroyd, 2013). A very important discovery was that a genetic overlap exists between legumes root nodule symbiosis and the more ancient arbuscular mycorrhizal symbiosis referred to as the "SYM" signaling pathway (also known as the CSSP; Capoen et al., 2009; Markmann and Parniske, 2009; see Chapters 54, 55, 108, 110). Very little is known about symbiotic determinants of actinorhizal plants (see Chapter 43). Apart from the recent demonstration of the role of *SymRK* and *CCamK* in different actinorhizal species (Gherbi et al., 2008; Markmann et al., 2008; Svistoonoff et al., 2013; Chapter 43, this book), exploration of genomic data developed using *A. glutinosa* and *C. glauca* allows to identify orthologs of most of legume genes covering all members of the symbiosis pathway from the signal perception to the nodulation process *via* the bacterial and fungal common part (Hocher et al., 2011a). Six of them (*Lys6*, *CCaMK*, *Hap2-1*, *HMG*, *Cyp2*, and *Cyp4*) were identified in both species. More interestingly, expression analysis of these genes revealed that transcript accumulation in nodules *versus* uninfected roots was not only comparable between the two actinorhizal species but also similar to those found in legumes (Hocher et al., 2011a). These results support thus, for the first time, a role for these genes in the

actinorhizal–*Frankia* symbiosis and suggest a conserved signaling pathway for endosymbiosis. Moreover, the overlapping of legume and actinorhizal root nodule symbioses reinforces the hypothesis of a common genetic ancestor of the nodulating clade with a genetic predisposition for nodulation (Soltis et al., 1995; Doyle, 2011).

42.1.9 A Conserved Genetic Program Among Endosymbioses

Some features of root nodule endosymbiosis could have been recruited from the more ancient arbuscular mycorrhiza (AM), which is formed by the majority of land plants with fungi belonging to the phylum Glomeromycota (Kistner and Parniske, 2002; Oldroyd et al., 2011). This led to the hypothesis that pre-existing AM genes were recruited during the evolution of root nodule symbiosis (Parniske, 2008).

Concerning actinorhizal species, information about arbuscular mycorrhizae is still very sparse (Tromas et al., 2012; Diagne et al., 2013). In order to identify the *C. glauca* genes regulated by AM symbiosis, the *Casuarina* 15K chip was hybridized with cDNA from two biological conditions: noninoculated roots and roots that are 45 days after inoculation by the fungus *Rhizophagus irregularis*. Global analysis resulted in the identification of a set of differentially expressed genes in *C. glauca* AM roots (Tromas et al., 2012). Comparative transcriptomics was thus used to identify genes induced during AM in several plant species, including the legume species *Medicago truncatul*a, rice and the actinorhizal tropical tree *C. glauca*. This analysis revealed a group of 84 genes induced during AM in these three distant species. Interestingly, these genes correspond mainly to proteases, cytochrome P450 family, and transporters, and more probably represent core functions needed for AM symbiosis.

Moreover, a group of genes commonly induced during AM, rhizobial, and actinorhizal nodulations was also identified by comparing the genes upregulated in AM and actinorhizal symbioses in *C. glauca* to those upregulated in both nodules and AM recently identified in the model legume *M. truncatula.* Twenty-four *C. glauca* genes induced in AM roots and nodules (MycUp/NodUp) presented significant sequence homology with *M. truncatula* MycUp/NodUp genes. Once again, genes encoding proteases formed the largest cluster (10/24), suggesting that proteases play a significant common role in the three endosymbioses. Gene encoding transporters represented the second largest group. These genes might represent part of the heart of endosymbioses, conserved between the ancestral AM symbiosis and the more recent legume rhizobial and actinorhizal symbioses. This list represents genes probably linked to processes such as nutrient exchange, infection, and intracellular accommodation of the microsymbiont, and reflects the molecular tinkering that took place during evolution of nodulation using parts of ancestral AM mechanisms. Further functional characterization of these genes is now needed to understand their precise role in the three different endosymbioses and to explain how they were recruited during the evolution of RNS.

42.2 CONCLUSION AND FUTURE DIRECTIONS

In conclusion, the recent development of genomic resources for actinorhizal plant species and *Frankia* represent a major step that allowed to progress in the identification of key symbiosis determinants. The identification of a "core" symbiotic program essential for root nodulation and endomycorrhization in legumes and actinorhizal species provide important information on a likely recycling of ancient AM genes for new function related to nitrogen fixation in these species. However, further functional studies are now needed to understand their precise role in the different endosymbioses.

The recent development of high-throughput sequencing technologies (i.e., RNA-Seq), offers new perspectives for large-scale transcriptomic analyses. Two RNA-Seq projects are actually developed in our group on the *C. glauca–Frankia* symbiotic system. The first project is funded by ANR (France), and RNA-Seq is used to identify very early induced genes linked to the symbiotic process. The second project is funded by DOE-JGI (USA) and is developed in collaboration with the Common Microbiology Lab (IRD, Dakar – Sénégal). All plants of Casuarinaceae are known to be salt tolerant and our aim is to understand mechanisms linked to salt tolerance by identifying genes linked to salt tolerance in *C. glauca*. This project is linked to revegetation projects developed notably in Senegal. Another important perspective is to make all actinorhizal plants and *Frankia* genomics data available through a WEB platform with the aim to integrate and distribute knowledge related to actinorhizal symbioses worldwide.

On a longer term, the basic knowledge obtained on legume–*Rhizobium* and actinorhizal symbioses may help define strategies to expand the capacity of nodulation to nonnodulating crop plants (Beatty and Good, 2011; see Chapter 108).

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Chapter 43

Molecular Biology of Infection and Nodule Development in *Discaria trinervis–Frankia* Actinorhizal Symbiosis

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43.1 INTRODUCTION

To cope with nitrogen deficiency, some plants evolved the capacity to form nitrogen-fixing root nodules in association with soil bacteria. This ability is restricted to two groups of plants: legumes and *Parasponia* (Cannabaceae) that interact with Gram-negative Proteobacteria collectively called rhizobia, and actinorhizal plants, a group of 220 species of Fagales, Cucurbitales, or Rosales, that interact with Gram-positive actinomycetes of the genus *Frankia* (Vessey et al., 2005). All these plants are phylogenetically related and clustered together in the Fabid clade, which also contains numerous nonnodulating taxons. However, nodulation is not an ancestral trait, it probably appeared independently 12–16 times during the evolution of Fabids (Doyle, 2011). Fabids appear to have evolved a predisposition to develop nitrogen-fixing nodules, which is not found in any other group of plants. The genetic basis of this predisposition is not well understood.

Molecular mechanisms leading to nodulation are particularly well studied in model legumes where key signaling molecules and major molecular determinants involved in nodule formation have been discovered. One of the most studied features is the Nod signaling pathway, which is activated by the specific recognition of bacterial Nod factors by plant LYSM receptor-like kinases (see Chapter 51). At least seven genes involved in the Nod pathway are also essential to establish arbuscular mycorrhizae, suggesting that legume nodulation recruited parts of the ancient "myc" program used by most plants to interact with arbuscular mycorrhizal fungi (Oldroyd, 2013). Genes of the common nod/myc pathway are also needed to form root nodules in *Parasponia* and actinorhizal plants (Op den Camp et al., 2011; Gherbi et al., 2008; Markmann et al., 2008; see also Chapter 42). However, the recent discovery of a *Bradyrhizobium* strain that does not rely on Nod factors to interact with its legume host (Giraud et al., 2007; see Chapter 28) unveiled an unexpected diversity of mechanisms used by plants to interact with nitrogen-fixing bacteria even within the legume family. Exploration of the molecular basis of nodule formation is therefore needed in different branches of nodulators to identify universal mechanisms and the genetic basis of the predisposition for nodulation found only in Fabids. This review deals with *Discaria trinervis*, an actinorhizal shrub native in northern Patagonia and its interaction with *Frankia*, which we began to compare at the molecular level with other nodulating plants.

43.1.1 *D. trinervis*: Classification, Distribution, and Symbiotic Specificity

D. trinervis (Fig. 43.1a), recently renamed *Ochetophila trinervis* (Medan et al., 2009) belongs to the family Rhamnaceae, which is included in the Rosales order. *D. trinervis* is mainly found on poor land gravel soils in xeric forests and steppe sites, often close to rivers or lakes (Fig. 43.1b) and is always nodulated and mycorrhized (Chaia et al., 2006a). Two other species, *Discaria chacaye* and *Discaria articulata*, grow in the same region and the three species coexist in

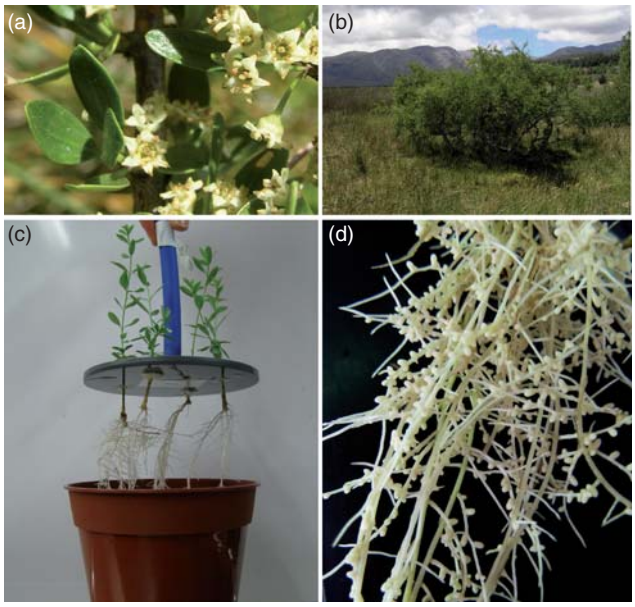

Figure 43.1 *Discaria trinervis* in its natural habitat, the northwest of Patagonia (a–b) and in hydroponic culture in laboratory conditions (c–d).

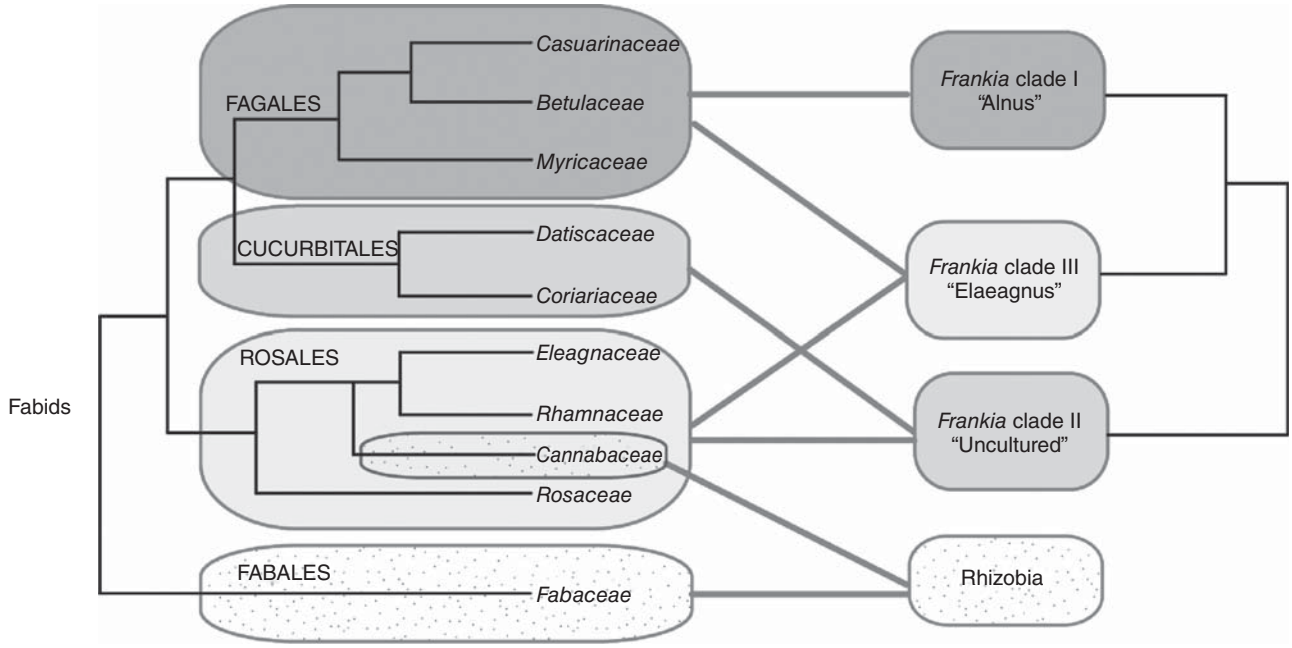

Figure 43.2 Phylogenetic relationships between plant families containing species able to form root nodules and the corresponding symbionts. Gray lines represent compatible interactions leading to nodulation.

some areas (Chaia et al., 2006b). Similar to all actinorhizal plants, *D. trinervis* shows symbiotic specificity, being nodulated by *Frankia* isolates from a particular set of actinorhizal plants belonging to Elaeagnaceae–Rhamnaceae clade, but not by *Frankia* from Myricaceae, Betulaceae, Casuarinaceae, Rosaceae, Coriariaceae, and Datiscaceae (Wall, 2000). *Frankia* isolates from Rhamnaceae and Eleagnacea belong to the same cluster (=clade III; Fig. 43.2), which appears to have the greatest metabolic versatility and are generally considered to be broad host-range symbionts with bigger genomes compared to *Frankia* from other clusters (Normand et al., 2007; see Chapter 24). Cross-inoculation experiments show that *Frankia* obtained from one *Discaria* species is able to nodulate all other *Discaria* with different levels of efficiency, suggesting that the recognition between *Frankia* and *Discaria* spp. would be mediated by a finely tuned signaling mechanism (Chaia et al., 2006b; Chaia, 1998).

43.1.2 Infection Establishment and Regulation

Frankia strains infect their hosts by two mechanisms: root hair infection as in *Myrica*, *Comptonia*, *Alnus*, and *Casuarina* or intercellular penetration as in *Elaeagnus, Hippophae, Ceanothus*, *Cercocarpus*, *and Discaria* (Wall, 2000). The initial steps of root hair infection are similar to what occurs in model legumes and have been extensively studied (Wall and Berry, 2008; Svistoonoff et al., 2010a). Much less is known about intercellular infection that takes place in about 35% of legume genera and 75% of actinorhizal genera (Sprent, 2007; Wall, 2000).

In *D. trinervis*, *Frankia* filaments in contact with the root surface adhere to the epidermis and enter the root by penetrating between epidermal cells and then between outer cortical cells (Fig. 43.3a). Root hairs are not involved in the initiation of nodulation and do not show any morphological response during these early interactions with the bacteria; they remain undeformed throughout the process of nodulation (Fig. 43.3a). Nevertheless, epidermal cells sense the presence of the invading bacteria and secrete locally an electron-dense material into the intercellular spaces through which *Frankia* filaments progress (Valverde and Wall, 1999b). Bacterial infection triggers cell divisions in the pericycle and the formation of a nodule primordium giving rise to a nodule lobe (Fig. 43.3a). Nodule lobes are structurally similar to a lateral root, with a central vascular bundle and an indeterminate apical meristem (Fig. 43.3a). *Frankia* filaments progress intercellularly until they reach the nodule cortex where they become intracellular as they infect cortical cells (Fig. 43.3a). No infection threads or transcellular infection could be detected, each infection takes place from the intercellular space (Valverde and Wall, 1999a). Mature nodules consist of several lobes with dichotomous ramifications and have an indeterminate growth (Fig. 43.3c).

Nodulation of *D. trinervis* is autoregulated (Valverde and Wall, 1999b; Valverde et al., 2000). Regulation by nitrogen (Valverde et al., 2000), phosphorous (Valverde

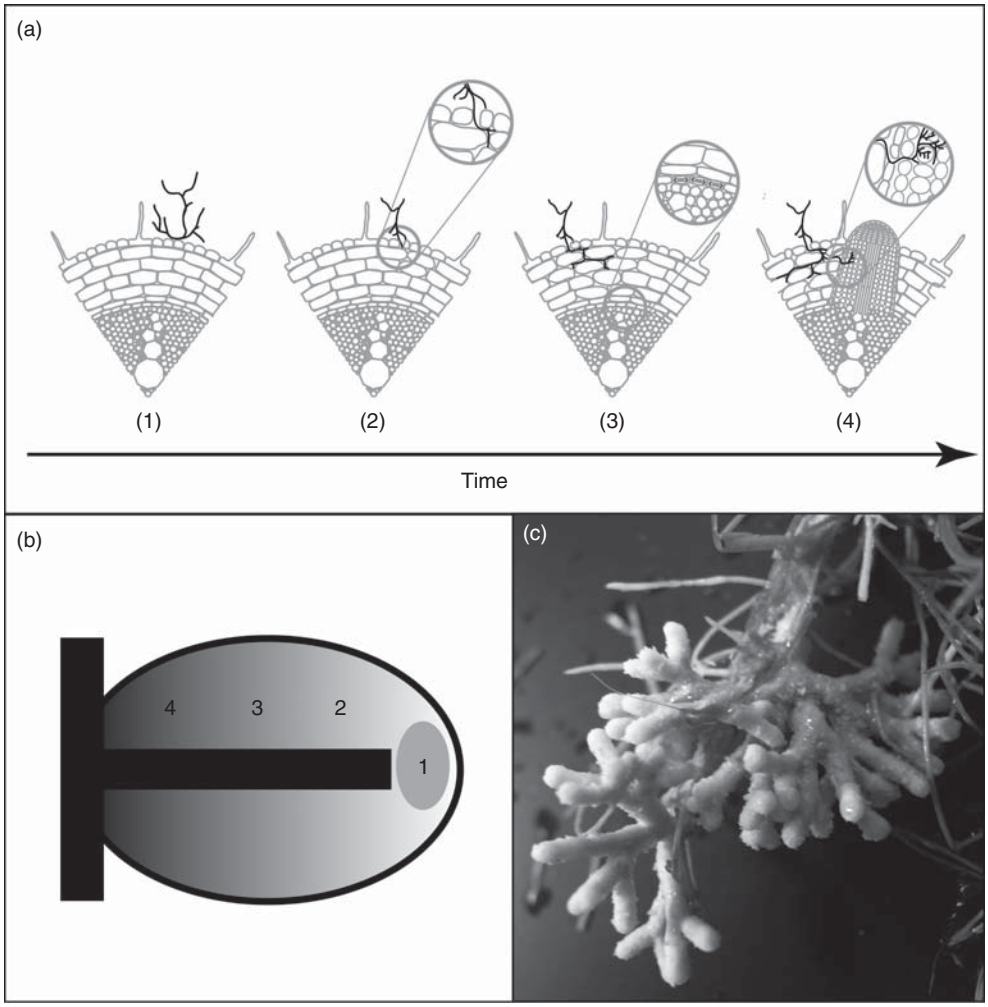

Figure 43.3 (a) Formation of actinorhizal nodules in *D. trinervis*. *Frankia* attaches to the root epidermis (1) and penetrates between epidermal cells without triggering any root hair deformation (2). Filaments progress in the intercellular space (2) triggering cell division in the pericycle (3) to form a nodule primordium (4). *Frankia* becomes intracellular in cortical nodule cells which are infected by filaments from the intercellular space. (b) In mature nodules, four zones are observed: the meristematic zone of the nodule that is always free of *Frankia* (1), the infection zone (2) containing newly formed cortical cells that are progressively infected and become hypertrophied, the fixation zone (3) where *Frankia* nitrogen-fixing vesicles are present, and a senescence zone (4) where Frankia and plant cells degenerate. The nodule presents a root-like structure with a central vasculature. (c) A mature nodule consisting of several branched lobes.

et al., 2002), and ethylene (Valverde and Wall, 2005) has been described and shows similar characteristics to what is observed in legumes and other actinorhizal plants, regardless of the infection pathway. *D. trinervis* autoregulation shows a particularity concerning mature nodules and control of new infections, compared to root hair-infected nodulated plants as *Alnus acuminata* (Chaia et al., 2006a,b). If mature nodules in *D. trinervis* stop to fix nitrogen for any reason, new infections by *Frankia* are not allowed in the growing younger parts of the root as it occurs in root hair-infected nodulated plants as *A. acuminata* (new infections can occur in this case). It is hypothesized that *Frankia* remaining intracellularly in the root cortex of *D. trinervis* sustain the feedback inhibition of new infections in the growing root. If more nitrogen fixation is needed because of the growth demand of the plant, new symbiotic tissue is developed by enlargement and further development of already existing mature nodules instead of generating new infections and development of new nodules – as it happens in intracellular root hair-infected plants (Chaia et al., 2006a,b). This mechanism found in laboratory experiments (Chaia et al., 2006a,b) appears to be corroborated by seasonal variations of field nodules of *D. trinervis* growing in the wild in Patagonia (Chaia, 1993).

Intercellular infection with similar characteristics to the one described in *D. trinervis* is a common feature of all

actinorhizal Rosales (Wall, 2000) and was recently described in mutants of *Lotus japonicus*, which can be nodulated by rhizobia unable to synthesize Nod factors, suggesting that intercellular infection is an ancestral process that is still present in some lineages (Madsen et al., 2010).

43.1.3 Establishment of Molecular Tools for *D. trinervis*

In laboratory conditions, *D. trinervis* plants grow vigorously and nodulate well in hydroponics (Fig. 43.1c and d). This is an advantageous system to study the symbiotic interaction because roots can be easily inoculated, observed, or extracted with minimal perturbation. Such roots are also clean, white, and poorly lignified; as a consequence, simple protocols for nucleic acid extraction including commercial kits originally developed for model plants such as *Arabidopsis thaliana* or *M. truncatula* can be directly applied to *D. trinervis*.

An essential tool to perform molecular analyses is the possibility to perform genetic transformation. Stable genetic transformation performed using *Agrobacterium tumefaciens* is available for two Casuarinaceae species (Svistoonoff et al., 2010a) and several nonactinorhizal Rosales (Gambino and Gribaudo, 2012). However, establishing an efficient genetic transformation procedure based on *A. tumefaciens* is often difficult since it requires both an efficient gene transfer protocol and the development of an *in vitro* regeneration method. An alternative method is based on *Agrobacterium rhizogenes*. Similar to *A. tumefaciens*, this bacterium is able to transfer genes of interest into the genome of the infected host plant, but in addition its T-DNA fragment carries genes driving auxin and cytokinin biosyntheses. This modified hormonal balance stimulates the formation of roots at the wounding site. Composite plants consisting of a nontransgenic aerial part and transgenic roots can thus be easily obtained in few weeks without any *in vitro* regeneration step (Benabdoun et al., 2011). To set up this genetic transformation system in *D. trinervis*, we first analyzed its susceptibility to two strains of *A. rhizogenes*: A4RS and ARqua1 carrying a plasmid with a 35S::GFP fusion to detect transgenic roots. The classic *in vitro* inoculation was compared to an *ex vitro* method reported to be successful in several plant species (Collier et al., 2005). The *ex vitro* method using Arqua1 was the best compromise to obtain good cotransformation efficiency (up to 80%) while minimizing the impact on root system architecture, which is very strong with the aggressive A4RS strain. The symbiosis was found to be fully functional in composite plants as revealed by nodulation tests, histological observations, and acetylene reduction assays. Cotransformed roots were specifically and efficiently nodulated with *Frankia*, and the resulting nodules were indistinguishable from nontransgenic nodules in terms of developmental timing, anatomy, nitrogen fixation, and feedback control by nitrogen (Imanishi et al., 2011).

43.1.4 *D. trinervis* Infection: Conserved and Divergent Molecular Mechanisms Compared to *C. glauca*

Over the past years, considerable amount of information has been produced on molecular mechanisms that determine nodule formation in *Casuarina glauca* (Svistoonoff et al., 2010a; Peret et al., 2009). We were particularly interested in the early steps of the interaction when bacterial recognition and accommodation take place. In contrast to *D. trinervis*, *Frankia* infects *C. glauca* via root hairs. As in model legumes, *Frankia* induces root hair deformation and penetrates a deformed root hair encapsulated by a cell wall deposit. Cell divisions are triggered in the cortex and give rise to a swollen structure, the prenodule, which becomes infected by *Frankia*. Similar to *D. trinervis*, cell divisions are induced in the pericycle and give rise a nodule primordium. Cortical nodule cells become invaded by *Frankia*. Infection in prenodules and nodules is mediated by infection threads, that is, lines of transcellular encapsulated hyphae (Peret et al., 2009). Several genes expressed specifically during the infection of *C. glauca* by *Frankia* were characterized, and their promoters were found to retain their specific activation pattern in model legumes indicating a strong conservation of molecular mechanisms involved in root hair infection between legumes and actinorhizal plants (Perrine-Walker et al., 2011). Three of these genes whose expression is strongly related to plant cell infection by *Frankia* were analyzed in the context of *D. trinervis* intercellular infection: *MtEnod11*, *Cg12*, and *CgAux1*.

MtEnod11 is a gene from *M. truncatula* coding for a proline-rich cell wall protein widely used as an early infection-related molecular marker for associations involving rhizobia, arbuscular mycorrhizal fungi, and even nematodes (Boisson-Dernier et al., 2005). Pro*MtEnod11* is strongly activated in *M. truncatula* and *C. glauca* during *Frankia* infection in infected root hairs, prenodules, and nodules, particularly in the infection zone (Svistoonoff et al., 2010b). *Cg12*, a gene from *C. glauca*, encodes a subtilisin-like serine protease expressed specifically in response to *Frankia* inoculation in infected root hairs, infected cells in prenodules, and mature nodules (Laplaze et al., 2000; Svistoonoff et al., 2003). In *D. trinervis*, Pro*MtEnod11* and Pro*Cg12* were found to be specifically activated in response to *Frankia* inoculation (Imanishi et al., 2011). Strong activation was detected in cells containing *Frankia* intracellularly in nodules as it is the case in *C. glauca* (Svistoonoff et al., 2003; Svistoonoff et al., 2010b) or *M. truncatula* (Svistoonoff et al., 2004; Boisson-Dernier et al., 2005) and also in some epidermal and cortical cells 3–7 days after *Frankia* inoculation. At this early stage, *Frankia* has not yet become intracellular; *Frankia* filaments are present exclusively in intercellular spaces and are progressing toward the future nodule primordia (Valverde and

Wall, 1999a). These results were later confirmed with the promoter of *Dt12*, the putative ortholog of *Cg12* in *D. trinervis*. The fact that these cells strongly activate Pro*Cg12* and Pro*MtEnod11* suggest the presence of at least a partial overlapping developmental program related to bacterial accommodation inside the root, which does not require the presence of intracellular bacteria.

CgAux1 encodes an auxin-influx carrier from *C. glauca*. Similar to Pro*Cg12* and Pro*Mtenod11*, Pro*CgAux1* is activated in *C. glauca* in *Frankia*-infected cells including root hairs, and also in root vasculature and root tips (Péret et al., 2007; Péret et al., 2008). In contrast to Pro*Cg12* and Pro*MtEnod11*, Pro*CgAux1* retains its nonsymbiotic activation pattern in *D. trinervis* but not its infection-related activation: expression was found in root tips and vasculature and nodules behaved as normal roots showing Pro*Aux1* activation only in the vasculature and meristem. As for *Cg12*, we confirmed these results with *DtAux1*, the putative ortholog of Cg*Aux1* in *D. trinervis* (Imanishi et al., 2014). These results point to a different auxin distribution in *D. trinervis* compared to *C. glauca*. Auxin distribution and fluxes were recently analyzed in *C. glauca* using antibodies against two auxins (PAA and IAA) and the auxin-efflux carrier PIN1 (Perrine-Walker et al., 2010). We performed similar experiments in *D. trinervis*. In *C. glauca*, auxins are concentrated in infected cells. The distribution of *CgAux1* in infected cells and PIN1 in noninfected cells surrounding infected cells is coherent with an accumulation of auxin in infected cells. Interestingly, in *D. trinervis*, PIN1 labeling was found in infected cells that are also labeled by anti-PAA and anti-IAA antibodies, again pointing to differences in auxin fluxes and roles between the two symbiotic systems (Imanishi et al., 2014).

43.1.5 An Universal Role of CCamK Activation in Nodule Organogenesis

In legumes, the *CCamK* gene encodes a calcium and calmodulin-dependent kinase, which plays an essential role in nodulation and mycorrhization (Oldroyd, 2013; see Chapter 54). Autoactive versions of CCAMK induce the formation of spontaneous nodules in model legumes (Gleason et al., 2006; Tirichine et al., 2006) and also in *Parasponia* (Op den Camp et al., 2011). We recently characterized the ortholog of *CCAMK* in *C. glauca*, *CgCCAMK*. We showed that *CgCCAMK* is essential for actinorhizal nodulation and mycorrhization in *C. glauca* and is able to fully complement the *M. truncatula* dmi3 mutant. A construct with a deleted version of *CgCCamK* lacking the autoinhibitory domain was introduced in *C. glauca*. Similar to legumes, this autoactive CgCCAMK lead to the formation of small spontaneous nodules in *C. glauca* without the presence of *Frankia*. The same construct was introduced in *D. trinervis* and in contrast to *C. glauca* nodules were numerous and bigger, almost identical to normal nodules except that they did not contain any *Frankia* (Svistoonoff et al., 2013). These results demonstrate that the activation of CCAMK is an universal mechanism, sufficient to activate the organogenesis program leading to nodule formation in all nodulating species, regardless of the symbionts or the infection mode.

43.1.6 Identification of Signaling Molecules and Their Receptors

As it was stated earlier, the interaction between *D. trinervis* and *Frankia* involves recognition between the symbiotic partners (Wall, 2000). Recognition should be a consequence of signal–receptor recognition events. Since there is no early plant response such as root hair deformation that can be used to bioassay early signaling between *Frankia* and *D. trinervis* roots, a different physiological approach measuring nodulation kinetics (Gabbarini and Wall, 2008) was used to characterize a diffusible factor of *Frankia* origin from strain BCU110501, isolated from *D. trinervis* (Chaia, 1998). This factor is relatively small (<12 kDa), it is synthesized by dense bacterial cultures, is negatively charged at pH 7.0, and is sensitive to protease treatments (Gabbarini and Wall, 2011a,b). Moreover, this diffusible *Frankia* factor appears to be related to the root hair deforming activity of *Frankia* BCU110501 on *A. acuminata* root hairs (Gabbarini and Wall, 2011a,b), suggesting a partial recognition plant response. It is worth noting that the barrier of symbiotic recognition between *Frankia* and either *D. trinervis* or *A. acuminata* could not be bypassed or physiologically complemented by just diffusible signals of plant or bacterial origin (Gabbarini and Wall, 2011a,b). To further characterize these signaling molecules, we plan to develop bioassays based on the specific activation of Pro*Dt12* at early stages of the interaction with *Frankia*.

In legumes, nod factors are perceived by receptor-like kinases containing LysM motifs encoded by genes *NFR5* and *NFR1* in *L. japonicus* (Oldroyd, 2013). We recently identified a putative ortholog of *NFR5* in *D. trinervis* (=*DtNFR5*) and will perform a functional analysis of this gene using an RNAi approach.

43.2 CONCLUSION

The development of molecular tools in *D. trinervis* has opened new possibilities to study the genetic mechanisms of intercellular root invasion and single cell infection. The study of genes previously characterized in other actinorhizal species such as *C. glauca* or in model legumes will provide useful information to understand this poorly known infection mechanism and allow the identification of universal mechanisms used by all plants able to form nitrogen-fixing nodules. *D. trinervis* is a Rosale, an order containing

numerous species unable to nodulate (most Rosaceae) and the only nonlegume able to form nodules with rhizobia (=*Parasponia*). Comparative studies at the molecular level between nodulating and nonnodulating Rosales should bring important cues about the evolution of nodulation.

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Chapter 44

Lotus japonicus Nodulates When It Sees Red

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44.1 INTRODUCTION

In nature, many legumes develop nodules on their roots in which rhizobia fix atmospheric nitrogen into ammonia by means of the enzyme nitrogenase. In return, the rhizobia obtain photoassimilates from the host that had been fixed within plant leaves, demonstrating that light quantity is critical for the effectiveness of this symbiosis. However, light quality also has a tremendous influence on the success of the legume–rhizobial symbiosis. In this chapter, we review the literature on the effects of light quality on the establishment of the legume–rhizobial symbiosis and describe our own work to update our current knowledge concerning nodule development and light.

44.2 RESULTS AND DISCUSSION

Numerous reports exist in the literature about sufficient light quantity being critical for effective root nodule formation (Fred and Wilson, 1934; Fred et al., 1938). In addition, adding glucose and sucrose to the nutrient medium when light was limiting enhanced root nodule formation in some legumes (Van Schreven, 1959). These observations support the idea that the accumulation of photoassimilates provides energy and compounds important not only for root nodule formation but also for nodule function to maintain a successful mutualism between the two symbiotic partners.

If we consider the fact that chlorophyll absorbs both red (R; 600–700 nm) and blue light (400–500 nm), we can conclude that the difference in light quality (wavelength) also affects the photosynthetic activity leading to the establishment of the symbiosis. The fact that the effects of light quality on symbiosis are independent of photosynthetic activity has been reported by a number of investigators. Lie (1964) studied the effects of short-time irradiation of R or far-red (FR) light at the end of the photoperiod of the light cycle of the day (end-of-day; EOD) on pea and kidney bean root nodule formation, and reported that root nodule formation following irradiation with FR light was suppressed compared to R light treatment (Lie, 1964). This phenomenon – that root nodule formation is suppressed by irradiating FR light at the EOD – was reported not only for pea (Lie, 1969) but also for broad beans (Lie, 1971), soybean (Kasperbauer et al., 1984; Hunt et al., 1987), and southern pea (cowpea) (Kasperbauer and Hunt, 1994). In contrast, Balatti and Montaldi (1986) showed that the number and biomass of soybean root nodules

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20 days after inoculation with *Bradyrhizobium japonicum* were significantly increased following FR irradiation in EOD compared to R irradiation. The reasons for these contradictory results are unclear, but may depend on light quantity or the exact timing of the irradiation. Nevertheless, root nodule formation was restored by the irradiation of R after FR treatment in EOD (FR + R) and repressed once again following a light treatment of FR + R + FR in EOD. Because these results illustrate the classic photoreversibility of FR with R, the involvement of phytochrome in root nodule formation was strongly indicated (Lie, 1964; Hunt et al., 1987; Kasperbauer and Hunt, 1994).

That phytochrome participates in the perception of the R/FR ratio and the shade avoidance syndrome (SAS) is well known. Plants have photoreceptors that sense the presence of their neighbors by monitoring the ratio of R, mainly absorbed by chlorophyll, to FR light, which is not. A low R/FR ratio indicates the presence of neighbors that may be competitors for photosynthetically active radiation (PAR), thereby initiating the SAS, such that plant crowding causes plants to grow taller or to bend to the light to avoid being shaded (Smith and Whitelam, 1997; Neff et al., 2000; Franklin, 2008; Franklin and Quail, 2010). However, these low R/FR light conditions are suboptimal for root nodule formation. Kasperbauer and Hunt (1994) investigated the effect of the difference of R/FR ratio on southern pea root nodule formation by covering the surface of the pots with either gray-white or brick-red soil. Because the spectral distribution of light reflected from different soils is not the same, the R/FR ratio radiated to each pot through its covering differed. The final results showed that the number of root nodules 18 days after inoculation was higher when plants were grown under high R/FR (gray-white soil) than under low R/FR (brick-red) conditions (Kasperbauer and Hunt, 1994). In another experiment, this time in the field where multiple plants were planted together, the R/FR light ratios that these plants perceived depended not only on the degree of shading but also on row orientation because chlorophyll absorbs R light more efficiently than FR light. Root nodule formation for soybean and southern peas planted in either a north–south (low R/FR) or east–west (high R/FR) orientation was investigated. For both species, plants positioned in an east–west row orientation produced more nodules than those orientated north–south. In other words, plants grown under high R/FR ratios produced more nodules (Hunt et al., 1990; Kasperbauer and Hunt, 1994). However, the photosynthetic photon flux density (PPFD) for plants exposed to high R/FR conditions was higher than that for low R/FR exposure in both sets of experiments. Therefore, these investigators did not eliminate the possibility that root nodule number was affected by photosynthetic activity and the greater allocation of photoassimilates. Based on these results, we reasoned that root nodule formation was linked to the perception of the R/FR ratio and used *Lotus japonicus* (Suzuki et al., 2011; Shigeyama et al., 2012) to test this hypothesis.

To verify that root nodule formation is under the control of the perception of the R/FR ratio, we examined nodule formation in *L. japonicus* MG20 wild-type and *phyB* mutant plants grown under different R/FR light conditions. In our study, the PPFD of the R light-emitting diode (LED) remained constant to eliminate differences in the amount of photosynthate produced under low R/FR and high R/FR treatments. Signaling via phyB, the major red light photoreceptor used during seedling development, regulates responses to photoperiodism, end-of-day FR, and R/FR ratios in white light-grown plants (Neff et al., 2000; Franklin et al., 2003). From our experiments, we determined that the shoot and root fresh weights of *phyB* mutants were significantly reduced compared to those of MG20 plants grown under white light conditions. In addition, the number of root nodules of the *phyB* mutants was lowered compared to that of MG20 plants. To better understand the effect of the R/FR ratio on plant growth, we measured the fresh weights of the aerial plant parts, root length, and root nodule number under different R/FR light conditions. We found no statistically significant difference for either root length or shoot fresh weight for *L. japonicus phyB* mutants or MG20 plants, respectively, when the plants were grown under high R/FR and low R/FR light conditions. However, very few nodules developed on the *phyB* mutant roots under different R/FR light conditions. In addition, although nodules developed on MG20 plants grown in high R/FR light, very few nodules were detected on the roots of the low R/FR-grown plants.

Sucrose, a highly soluble disaccharide that provides energy sources for plant cells (Huber, 1989), is synthesized from the primary products of photosynthesis. To investigate whether a difference in photosynthetic activity was present while PPFD is constant, the sucrose content of the roots was measured after the plants were grown under different R/FR light conditions. We found that the sucrose content of roots in low R/FR-grown plants increased compared to the high R/FR-grown plants, suggesting FR light irradiation may be responsible for the increase in sucrose content. Nevertheless, the fact that root nodule number was reduced in the low R/FR-grown plants in spite of the higher sucrose content strongly suggests that phyB sensing of the R/FR ratio has a significant influence on nodulation. However, we cannot completely eliminate the possibility that photosynthetic products contribute to root nodule formation when the plants are grown under different R/FR light conditions.

Because the number of root nodules for both low R/FR-grown MG20 plants and white light-grown *phyB* mutants decreased, we hypothesized that phyB-mediated signaling in plants controlled by root nodule formation. Robson et al. (2010) suggested that phyA and JA signaling cooperate to regulate the balance between shade avoidance responses in FR-enriched light and defense responses

to mechanical damage or herbivores. In low R/FR light, phyB signaling suppressed both JA-mediated gene expression and JA-dependent defenses against insect herbivory (Moreno et al., 2009). By contrast, other reports indicate that antagonistic interactions between JA-mediated defense signaling and chromophore-mediated light signaling exist. For example, mutations in either *HY1* or *HY2*, which encode a phytochromobilin synthase, enhanced JA production and sensitivity (Zhai et al., 2007). For root nodule formation, JA has been reported to be a negative regulator. In *L. japonicus*, shoot-applied methyl jasmonate (MeJA) strongly suppressed the early stages of nodulation, including infection thread formation and *NIN* expression, and also inhibited lateral root formation (Nakagawa and Kawaguchi, 2006). In *Medicago truncatula*, JA also suppressed root nodule development, at an even lower concentration (Sun et al., 2006). Thus, we predicted that JA production and/or sensitivity would be enhanced in low R/FR-grown MG20 plants and white light-grown *phyB* mutants. We analyzed the expression of JA-responsive genes (*PDF1.2*, *JAR1*, and *MYC2*) in *L. japonicus* grown under different R/FR light conditions. The expression of *PDF1.2*, *JAR1*, and *MYC2* genes decreased in low R/FR-grown MG20 plants compared with plants grown under high R/FR light conditions. Moreover, their expression levels declined in *phyB* mutants under white light conditions in contrast to MG20 plants. These results strongly suggested that JA production and/or sensitivity decreased in low R/FR-grown MG20 plants and white light-grown *phyB* mutants, which was contrary to our expectations. To check whether JA concentration correlated with the expression of JA-responsive genes, we measured the endogenous concentrations of JA and JA-Ile in roots grown under white light condition. We found no significant difference in endogenous JA concentration between MG20 wild-type plants and *phyB* mutant plants. However, the endogenous concentration of JA-Ile significantly decreased in roots of *phyB* mutants. JA-Ile, the isoleucine conjugate of JA, is a biologically active hormonal signal (Staswick and Tiryaki, 2004) and is generated by a JAR1-encoding JA-amino acid synthetase. The *JAR1* gene product is involved in pathogen defense, sensitivity to ozone, and wound responses (Guranowski et al., 2007; Staswick, 2008; Koo et al., 2009). Our results might be explained by the fact that the conversion of JA to JA-Ile was suppressed due to decreased *JAR1* expression in the *phyB* mutants grown under white light conditions, which would give rise to a reduced concentration of JA-Ile in the *phyB* mutants. Thus, we concluded that root nodule formation was suppressed because the JA-Ile concentration was low in plants grown under low R/FR light conditions.

To examine the effect of JA directly on root nodule formation under different R/FR light conditions, JA was added to the plant growth medium. Both shoot and root growth of MG20 plants were decreased as the concentration of JA increased. Although the root nodule number per plant was reduced after adding a high concentration (10 μM) of JA, nodule number per plant for plants grown in low (0.1 μM) JA significantly increased when compared to control plants that received no exogenous JA. Furthermore, nodule number per root length increased for plants treated with 0.1, 1, and 10 μM JA over the untreated controls. Our results differed from those reported for *M. truncatula* (Sun et al., 2006), in that in our study, we found that *L. japonicus* nodule number was increased at low concentrations of JA.

If reduced nodule formation in low R/FR-grown MG20 plants and white light-grown *phyB* mutants is due to a low concentration of endogenous JA-Ile, we predicted that nodule formation would be enhanced by JA application. To test this possibility, the effect of JA treatment on root nodule development was analyzed. We found that the number of root nodules and nodule primordia in 0.1 μM JA-treated plants was slightly increased compared to untreated plants. We also examined the expression of JA-responsive genes and the *NIN* gene in 0.1 μM JA-treated or untreated plants. *NIN* is required for infection thread formation and nodule primordium initiation in *L. japonicus* (Schauser et al., 1999). The expression of *NIN* gene was significantly increased in 0.1 μM JA-treated plants. Although JA addition did not affect the total root length, the number of infection threads per root length was significantly increased in MG20 plants compared to the untreated plants. These results strongly suggest that low concentrations of JA function as a positive regulator for root nodule formation in *L. japonicus*.

In addition, we analyzed the effect of JA treatment on root nodule formation in *phyB* mutants. Following 0.1 μM JA treatment of *phyB* mutants, shoot length was unaffected and root length was decreased compared to the untreated plants. However, the number of root nodules per plant significantly increased compared to the number in the untreated plants. Moreover, nodule number per root length significantly increased in response to 0.1 and 1 μM JA treatments. Based on the responses of JA treatments to shoot length, total root length and root nodule number, the sensitivity of MG20 plants and *phyB* mutants to JA is not likely to be significantly different. To summarize, the cause of reduced root nodule formation in low R/FR-grown MG20 plants and white light-grown *phyB* mutants is due to the inhibition of JA-Ile production.

The literature also reports about the effects of R/FR ratios on the interaction between plants and microbes. For example, low R/FR ratio reduced *Arabidopsis* resistance to *Botrytis cinerea* (Cerrudo et al., 2012),‘ and fluorescent illumination with a high R/FR ratio improved the resistance of cucumber seedlings to powdery mildew (*Sphaerotheca cucurbitae*) (Shibuya et al., 2011). Moreover, in the case of the interaction between plants and pests, wild tobacco and *Arabidopsis* grown under high R/FR conditions were less susceptible to feeding damage by insect herbivory compared to low R/FR-grown plants (Izaguirre et al., 2006; Moreno

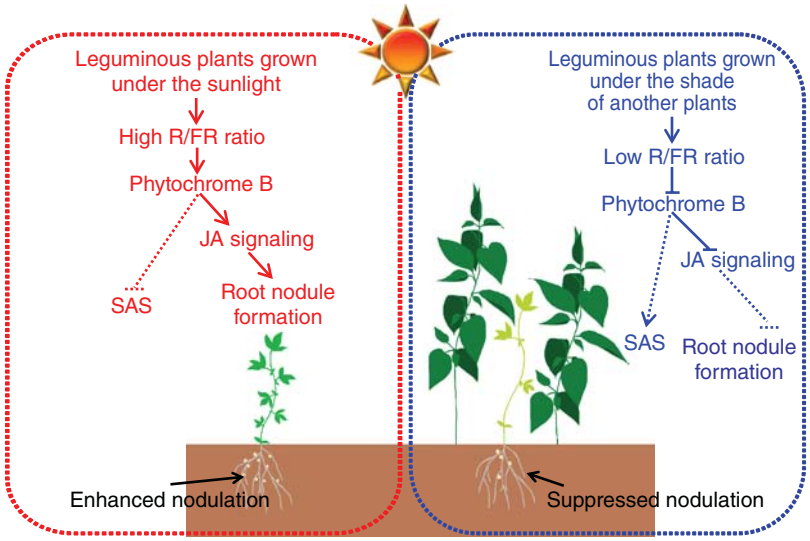

Figure 44.1 Model representing the proposed mechanism of JA and phyB signaling for shade perception and root nodule formation. In high R/FR light, phyB suppresses SAS and enhances root nodule formation through an increased concentration of JA-Ile. In low R/FR light, SAS is restored by the inactivation of phyB, and root nodule formation is suppressed due to a reduced concentration of JA-Ile. Small letters and dotted lines mean inactivation and suppression, respectively.

et al., 2009). JA signaling is involved in these plant defense mechanisms. Plants grown under high R/FR light produced more JA and protected themselves from pathogens, whereas plants grown under low R/FR light were more susceptible to infection or feeding damage by pathogens and predators due to the reduced production of or decreased sensitivity to JA (Cerrudo et al., 2012; Moreno et al., 2009; Ballaré, 2011). In root nodule symbiosis, the rhizobial infection process is enhanced by this mechanism.

For plants, photosynthesis is the most important biochemical reaction for survival. Plants grow taller or bend to light, but at the same time they assume the risk that they are more likely to be invaded by pathogens. In low R/FR light, phyB is inactivated by photoconverting the active Pfr form into Pr, and as a result, phytochrome-interacting factor 4 (PIF4) and 5 (PIF5) accumulate, leading to the synthesis of auxin (IAA) and bioactive gibberellins (Ballaré, 2011), phytohormones required for plant growth. However, the synthesis of phytohormones is incompatible with the production of JA, making it very difficult for plants to maintain active growth and high-level defense responses at the same time. Legumes show the same energy constraints by limiting the initiation of energy-costly nodules under low R/FR conditions. However, by obtaining sufficient nitrogen through the establishment of a symbiosis with rhizobia for maintaining effective growth, legumes may ultimately be better able to balance growth and defense than nonlegumes.

A model representing the proposed mechanism of JA and phyB signaling for shade perception and root nodule formation is depicted in Figure 44.1.

In high R/FR light conditions, phyB suppresses SAS and enhances root nodule formation through an increased concentration of JA-Ile. In contrast, in low R/FR light conditions, SAS is restored by the inactivation of phyB and root nodule formation is suppressed due to the reduced concentration of JA-Ile. Our data show that root nodule formation involves the perception of the R/FR light ratio and requires signaling through phyB and JA. Although root nodule development is initiated in the soil under low light, the R/FR light conditions in soil are suboptimal for sustaining root nodule function. Thus, host legumes shaded by other plants initiate a shade avoidance response and modify their growth to obtain sufficient light for maximizing photosynthesis. Nevertheless, under such low R/FR light conditions, the host plants suppress root nodule development to conserve energy. We conclude that this SAS for root nodule formation is required for *L. japonicus* nodule development. In conclusion, sensing both light quality and quantity is essential for establishing and maintaining a successful nitrogen-fixation symbiosis. How common this interaction is among the symbiotic Fabaceae is not known at this time.

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Chapter 45

Out of Water of a New Model Legume: The Nod-independent *Aeschynomene evenia*

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45.1 INTRODUCTION

The pantropical genus *Aeschynomene* belongs to the *Dalbergieae* tribe, an important group within the papilionoid legumes that is represented by peanut (*Arachis hypogaea*), the second most important crop legume. They share with arachid and other legumes of both the Dalbergioid and Genistoid clades to be infected by rhizobial strains in an intercellular manner to produce fully infected nitrogen-fixing nodules.

The initial interest of studying *Aeschynomene* relies on the fact that 22 semi-aquatic species have the capacity to form nitrogen-fixing nodules on both roots and stems. This unusual behavior among legumes is only shared with a few species of the genera *Sesbania*, *Neptunia*, and *Discolobium* (Boivin et al., 1997). In addition, some *Bradyrhizobia* isolated from *Aeschynomene* stem nodules exhibit the property uncommon among rhizobia of developing a photosynthetic system (Evans et al., 1990; Giraud and Fleischman, 2004; see Chapter 28). It has been shown that the photosynthetic activity of *Bradyrhizobium* plays a key role during stem nodulation by directly furnishing energy to the bacterium that can be used for biological nitrogen fixation (Giraud et al., 2000; see Chapter 28).

More recently, some photosynthetic *Bradyrhizobium* strains have been shown to be unique because they lack the canonical *nodABC* genes required for the synthesis of Nod factors (NF), whereas they maintain the ability to elicit efficient nodules (Giraud et al., 2007). This revealed the existence of a Nod-independent symbiotic process. To identify the plant determinants of this new and original symbiotic process, *Aeschynomene evenia* was recently proposed as a new legume model. In this review, we discuss the interests of studying this species, its characteristics, and the development of functional tools for its analysis.

45.1.1 Interests of *A. evenia* as a New Model Legume

Studies of *Rhizobium*–legume symbiosis have mainly been conducted on the symbiotic models: *Lotus japonicus–Mesorhizobium loti*, *Medicago truncatula–Sinorhizobium meliloti*, and *Glycine max–Bradyrhizobium japonicum*. They have led to a better understanding of the molecular mechanisms of this interaction with the identification of numerous components of the symbiotic pathways (reviewed in Oldroyd et al., 2011 and Oldroyd, 2013). In these systems, the early steps of the symbiosis are well conserved: this involves the perception by the plant of bacterial signal molecules called Nod factors that trigger two coordinated programs, bacterial infection through transcelullarly progressing infection threads and distant nodule organogenesis (see Chapter 51). However, these model legumes belong to the Phaseolid and Galegoid clades, and they are far from representing the diversity of the symbiotic mechanisms used by legumes to interact with rhizobium (Sprent, 2007; Sprent and James, 2008) (Fig. 45.1). Indeed, legumes of the more basal Genistoid and Dalbergioid clades, which represent

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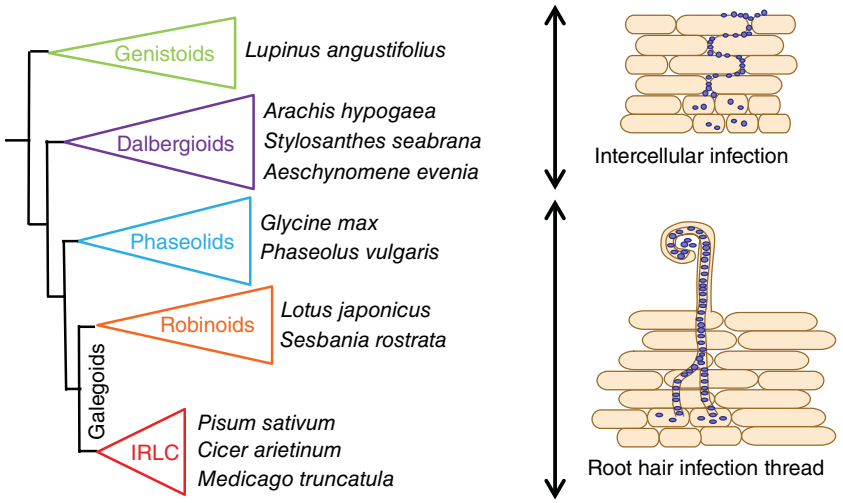

Figure 45.1 Evolution of the symbiotic infection process within the Papilionoid lineage. On the left, tree representation of the phylogeny of the Papilionoids with triangles representing the major clades. Some notable species are indicated for each clade. On the right, infection processes encountered in the different clades: the intercellular infection mode with progression of the rhizobia between root cells and the intracellular infection mode mediated by the development of an infection thread. (*Source*: Figure adapted from Bertioli et al. (2009) and Held et al. (2010).)

about 25% of the legume species, display another mode of infection where the entry of bacteria occurs intercellularly between epidermal cells. In addition to the absence of infection thread formation, there is no distant induction of a primordium. Instead, the nodule originates from successive divisions of only one or few infected cortical cells. This has been well described for *Arachis hypogaea* and *Lupinus albus*, two major legumes crops, as well as *Aeschynomene* spp. (Chandler, 1978; Chandler et al., 1982; Gonzales-Sama et al., 2004; Bonaldi et al., 2011).

Most surprisingly, in some *Aeschynomene* spp., a Nod-independent symbiotic process has been described where the symbionts, that is, photosynthetic *Bradyrhizobium* strains, are able to efficiently form nodules in the absence of NF synthesis (Giraud et al., 2007). A similar Nod-independent infection process has been observed in some double mutants of *L. japonicus*, although at a very low frequency (Madsen et al., 2010). In this genetic experimental system, single-cell infections were observed, without the formation of infection threads, independently of plant NF receptors and bacterial NF. This infection process was suggested to correspond to the ancestral state of the rhizobial infection, which could have been maintained during evolution in some legumes such as *Aeschynomene* spp. The absence of the requirement of NF to trigger symbiosis has been identified to date only in some *Aeschynomene* species, and the *Bradyrhizobium* strains that can use a Nod-independent infection strategy form a distinct lineage within the *Bradyrhizobium* phylogeny (Miché et al., 2010). The specificity between Nod-independent *Aeschynomene* and some *Bradyrhizobium* suggested to Okubo et al. (2012) that this symbiotic interaction could correspond in fact to an evolved state.

Deciphering the molecular mechanisms of the Nod-independent symbiosis will shed light on the evolutionary history of the *Aeschynomene–Bradyrhizobium* system. This would also serve to better understand the molecular basis of intercellular bacterial invasion and nodule organogenesis, particularly in Dalbergioid legumes, for which our knowledge remains limited due to the absence of appropriate genetic tools.

For these purposes, we explored the diversity of the Nod-independent *Aeschynomene* species. We selected *A. evenia* as it was closely related to the widespread but polyploid *A. indica* – used as host plant to study the symbiotic photosynthetic *Bradyrhizobium* – and it satisfied the required features for genetic and molecular approaches (Fig. 45.2) (Arrighi et al., 2012; Okubo et al., 2012; Bonaldi et al., 2010b). The *A. evenia* cultivar line IRFL 6945 displays a small and diploid genome (460 Mb, $2n = 20$) that is comparable to the two model legumes *M. truncatula* and *L. japonicus* (520 and 470 Mb, respectively). The species is autogamous, and an artificial hybridization method was successfully set up to perform directed crosses. *A. evenia* is easily cultured both *in vitro* and in pots. Efficient germination and small-sized plantlets facilitate their manipulation in laboratory conditions. A short generation time that can be accomplished in 10 weeks and the profuse seed production (thousands seeds over a plant lifetime) are other advantageous characteristics to obtain abundant material. Moreover, it is a short-lived perennial and rooting of cuttings is easily performed, which allows maintaining plants of interest. With genetic, symbiotic, and transformative properties that are discussed hereafter, this makes *A. evenia* a genetically tractable species to decipher the Nod-independent process.

45.1.2 Genotype Diversity

A. evenia is primarily American with a distribution from the southern United States to Argentina, but accessions are also found in regions of Africa. To investigate the intraspecific diversity, a set of 27 accessions representing almost all the

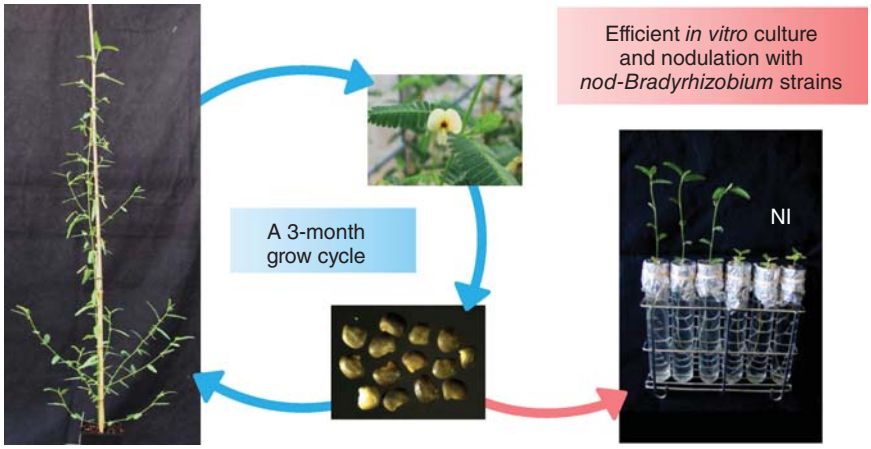

Figure 45.2 *Aeschynomene evenia* development and culture. (a) The plant presents a 3-month seed-to-seed cycle in greenhouse culture. (b) *In vitro* culture of inoculated plants (on the left) and not inoculated plants (NI) (on the right) with *Bradyrhizobium* strains. (*Source*: Adapted from Arrighi et al. (2012).)

accessions available in germplasm banks, and originating from regions of the world where this species naturally grows, was used. We conducted a polyphasic analysis by combining phenotypic, genotypic, molecular, and cross-compatibility approaches. It evidenced an important intraspecific differentiation with the accessions of the two described botanical types *evenia* and *serrulata*, forming sister taxa in the molecular phylogenies and showing a high genetic divergence. Such ongoing speciation process was also observed for other legume species such as *Arachis hypogaea* (Ferguson *et al*., 2004) and *Medicago truncatula* (Ellwood et al., 2006). This led us to propose a revision of the taxonomic classification of *A. evenia* (Arrighi et al., 2013).

In this new classification, *A. evenia* comprises two subspecies: *A. evenia* ssp. *evenia* and *A. evenia* ssp. *serrulata* that are morphologically well differentiated (Fig. 45.3a). The *evenia* subspecies is characterized by mostly glabrate stems, tender green leaves, and flowers with an elliptic standard petal. The *serrulata* subspecies differs being glandular in all parts. Leaflets are consistently ciliate-serrulate and dark green. Flowers present a rounded standard petal. Molecular analyses were performed using either the nuclear ribosomal internal transcribed spacer (ITS) sequence that is usually used for species identification or single nuclear gene-coding sequences. They indicated that accessions of the *evenia* and *serrulata* subspecies form distinct but related clades compared to the other diploid *Aeschynomene* species. In addition, flow cytometry analysis revealed a significant genome size difference between the two subspecies, ranging from 0.82 to 0.87 pg/2C for the *evenia* subspecies and between 0.94 and 0.99 pg/2C for the *serrulata* subspecies (Fig. 45.3a). Finally, using the artificial hybridization protocol successfully developed for *A. evenia*, the two subspecies were tested for their genetic compatibility. Inter-subspecies crossings were only possible when using the *evenia* subspecies as maternal parent. However, the F1 plants obtained, although highly flowering, were almost entirely sterile, producing only occasionally one-seed-bearing pods (Fig. 45.3a).

Each subspecies was, in turn, divided in two varieties (Fig. 45.3b). The *evenia* subspecies groups var. *evenia* and var. *pauciciliata*. The *evenia* variety is characterized by early-flowering accessions that tend to develop one primary axis with small axillary axes bearing the flowers. The *pauciciliata* variety comprises late-flowering accessions with developed secondary axes. Some accessions present moderately denticulate and ciliate leaflets suggesting to Rudd (1955) that they correspond to intergradations with the *serrulata* subspecies. In the latter, the var. *major* that includes the cultivated IRFL6945 line differs from the var. *serrulata* by larger plants developing bigger flowers. The genetic diversity between and within each subspecies was evaluated using RAPD and SSR markers. They gave congruent results in that the *evenia* and *serrulata* profiles were significantly different, and that variety delimitation on the basis of morphological and molecular markers were concordant. Interestingly, the polymorphism levels obtained using 82 EST-SSRs were similar: 24–33% between the two varieties of the *evenia* subspecies and 31–37% between the two varieties of the *serrulata* subspecies, whereas it rose up to 72–82% between the two subspecies (Fig. 45.3a,b). Contrarily to what was observed with the inter-subspecies hybridizations, bidirectional crossings of the two varieties for each subspecies could be efficiently performed and allowed to produce fully fertile hybrids (Fig. 45.3b). This indicates that crossing-compatibility was normal within each subspecies.

This knowledge on the phenotypic and genetic diversities present in *A. evenia* allows its judicious exploitation to develop genetic approaches. In particular, for map development, the use of parental lines belonging to the same subspecies is required, but fortunately the polymorphism level encountered in each subspecies appears to be sufficient. Characteristics found in *A. evenia* ssp. *evenia* such as a smaller genome size, a shorter growth cycle, a uniaxis habit, and interesting symbiotic features make up the difference. We therefore suggest this subspecies to be the most

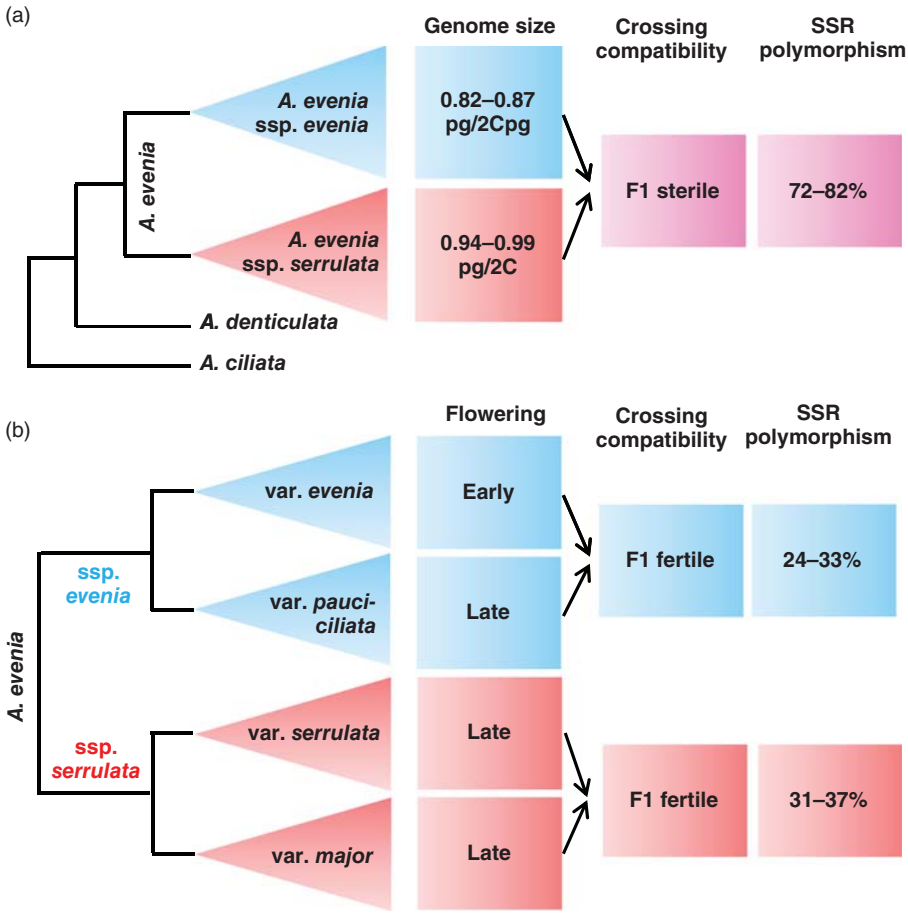

Figure 45.3 Genetic differentiation within the *Aeschynomene evenia* species. Schematic representation of the genetic diversity at the subspecies level (a) and variety level (b) with genome size and flowering characteristics, crossing compatibility, and SSR polymorphism. (*Source*: Adapted from Arrighi et al. (2013).)

appropriate for future projects of forward genetics (Arrighi et al., 2013).

45.1.3 Nodulation Characteristics

Similar to other semi-aquatic *Aeschynomene* species, *A. evenia* is endowed with the capacity to develop both root and stem nodules upon inoculation with photosynthetic *Bradyrhizobium*. It is efficiently nodulated with the commonly used laboratory strains ORS278 (using a Nod-independent strategy) and ORS285 (using both Nod-dependent and Nod-independent strategies) (Arrighi et al., 2012). Importantly, the genomes of these two strains have been completely sequenced and extensive collections of *Tn5* mutants have been developed (Bonaldi et al., 2010a; Giraud et al., 2007). In addition, *Bradyrhizobium* strains with reporter genes such as *GFP* and *GUS* are also available, thus facilitating the detailed observation of the symbiotic infection process (Bonaldi et al., 2011).

Stem nodulation has previously been analyzed in different *Aeschynomene* species, in particular *A. afraspera* (Alazard and Duhoux, 1987; 1988; Boivin et al., 1997). Like other *Aeschynomene* lines, accessions of *A. evenia* present stems with vertical rows of dormant root primordia. The ability of the root primordium to develop into an adventitious root can be easily checked by immerging cutting of stems in water (Arrighi et al., 2012). The root primordia on *A. evenia* have been reported to be less developed than those of *A. afraspera*, as they usually do not break the stem epidermis and just underlie it, sometimes at the proximity of lenticels. As a consequence, they are less prone to be infected by *Bradyrhizobium* (Alazard and Duhoux, 1987). But in the condition of high humidity, primordium dormancy can be broken, thus activating some nodulation sites. On *A. evenia* ssp. *evenia* stems, round swellings are rapidly visible (5–7 days) after inoculation with the strain ORS278, leading to hemispherical nodules with a broad attachment to the stem (Fig. 45.4a). Cross sections of mature nodules reveal a dark green peripheral cortex and a red-pigmented inner zone filled with *Bradyrhizobium* that is organized around the central vascular connection of the root primordium. The longevity of such nodules is no more than 9 weeks (Fig. 45.4a). On the contrary, stem nodules of *A. evenia* ssp. *serrulata*, which

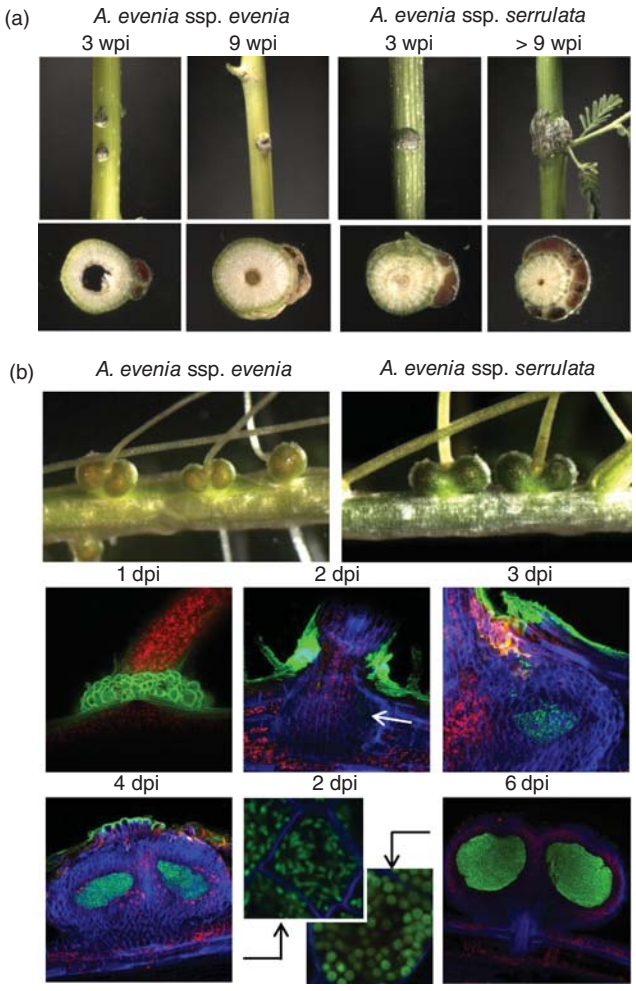

Figure 45.4 Stem and root nodulation in *Aeschynomene evenia*. (a) Stem nodules at two ages (3 and 9 weeks post-inoculation with the *Bradyrhizobium* strain ORS 278) in *A. evenia* ssp. *evenia* and ssp. *serrulata*. Below, cross-sections reveal the structure of the stem nodules. (b) Root nodules (15-day post in post-inoculation with the *Bradyrhizobium* strain ORS 285-*GFP*) showing similar morphology in both *A. evenia* subspecies. Below, kinetics of the infection and nodulation processes detailed for the *serrulata* IRFL6945 line. (*Source*: Confocal pictures reprinted from Arrighi et al. (2012) with permission.)

appear to be less frequent, mostly differ in their organogenesis. They tend to progressively form collars around the stem that embrace several root primordial vascular connections (Fig. 45.4a). These stem collar nodules could result from maintenance of mitotic activity in the peripheral cells of the nodule primordium leading to an important longevity (>3 months). It is therefore worth noting that there are significant variations in the stem nodule organogenesis between the two *A. evenia* subspecies. The *evenia* hemispherical nodules are similar to those of *A. afraspera*, whereas the *serrulata* collar nodules are reminiscent of those observed in *A. sensitiva* (Alazard and Duhoux, 1987; 1988; Boivin et al., 1997, Giraud et al., 2000).

Different from stem nodules, root nodules in both *A. evenia* subspecies were found to develop similarly at the base of lateral roots and to display the same spherical shape (Fig. 45.4b). The nodulation process was analyzed for the *serrulata* line IRFL6945 using the ORS285-*GFP* strain (Fig. 45.4b) (Arrighi et al., 2012). Shortly after inoculation, a strong proliferation of the bacteria was observed, specifically at the surface of the axillary root hairs surrounding the lateral roots. Then they penetrated between root hairs and progressed to the root cortex, most probably by successive collapses of the initially infected cells. At 2 dpi, some deeper infected cortical cells started to divide, giving rise to the nodule primordium. By successive divisions of these founder cells, the bumps rapidly evolved to spherical nodules. Between 5 and 6 dpi, the internalized *Bradyrhizobium* differentiated to bacteroids. This step was marked by a change from a rod to hemispherical shape (Fig. 45.4b) and the apparition of nitrogenase activity. Between 7 and 10 dpi, the nodules reached their mature size and displayed the classical histological organization of the aeschynomenoid nodules, that is, a large central zone of infected tissue surrounded by an uninfected nodule cortex comprising vascular bundles (Fig. 45.4b).

The process of symbiotic infection and of nodule organogenesis was found to be very similar to the one described for the related *A. indica* (Bonaldi et al., 2011). This suggested that the root nodulation process is well conserved among the Nod-independent *Aeschynomene* species, and that the use of *A. evenia* as symbiotic model is relevant to study the Nod-independent infection process.

45.1.4 Tools for Functional Analysis

Plant transformation is a prerequisite for the molecular and physiological analyses of *Aeschynomene* symbiotic gene function. However, transformation with *Agrobacterium tumefaciens* is a difficult and time-consuming approach, which is not suitable for high-throughput analysis of candidate genes. In the case of root tissues, where the nitrogen-fixing symbiosis takes place, the *Agrobacterium rhizogenes*-mediated transformation provides a convenient and rapid alternative and has been successfully developed for a number of legumes, such as *Medicago truncatula*, *Lotus japonicus,* and *Sesbania rostrata* (Boisson-Dernier et al., 2001; Kumagai and Kouchi, 2003; Van de Velde et al., 2003). For these reasons, we retained the option of an *A. rhizogenes*-mediated root transformation protocol for *Aeschynomene* with the goal to gain insights into the host genetic program recruited during the Nod-independent symbiotic association. The capacity to induce transgenic roots was initially investigated on *A. indica* that is used as

a convenient host plant. Two different wild-type *Agrobacterium rhizogenes* strains (A4RS and ARqua1) both carrying the *CaMV35S::GFP* construct and two infection methods were tested (stab inoculation or direct application of *Agrobacterium rhizogenes* on freshly sectioned radicles). The ARqua1 strain and infection by hypocotyl cutoff were retained and were subsequently used to determine the frequency of appearance of transformed roots and nodules in the new model legume *A. evenia* (Fig. 45.5a–c) (Bonaldi et al., 2010b; Arrighi et al., 2012). We are now able to transform *A. indica* and *A. evenia* with efficiencies of cotransformation of 80% and 60%, respectively, and an average nodulation efficiency of over 70% in less than 2 months (for comparison, the nodulation efficiency is 40% of transformed roots of *Casuarina glauca* and 90% of those of *Arachis hypogaea*) (Diouf et al. 1995; Sinharoy et al., 2009). In parallel, the expression pattern of the *CaMV35S* promoter was investigated in transgenic roots and nodules with the goal to determine whether its pattern was suitable for a gene expression downregulation approach in *Aeschynomene* symbiotic tissues. In *A. indica* and *A. evenia* transgenic nodules, the *CaMV35S* promoter was expressed in all cell types, including the infected cells of the central region, while in roots, 35S-driven *GUS* expression was restricted to vascular tissues, root tip, and root cortex (Fig. 45.5d,e) (Bonaldi et al. 2010b; Arrighi et al. 2012). According to *CaMV35S* promoter expression patterns and RNA silencing experiments described in other legumes (Gherbi et al. 2008; Kumagai et al., 2006; Sinharoy et al. 2009), those results are consistent with the achievement of functional analysis of symbiotic candidate genes.

One of the main issues raised by the discovery of the absence of *nodABC* genes in some *Bradyrhizobium* strains naturally associated with *Aeschynomene* species is the existence and nature of the rhizobial signal molecules required for this symbiosis. To address this question, we used a fusion of the *MtENOD11* promoter with a reporter gene as a bioassay. MtENOD11 is an early nodulin whose expression pattern has been widely described and used as marker of pre-infection and infection events occurring either during nitrogen-fixing and AM symbioses (Journet et al., 2001). During the *Medicago truncatula–Sinorhizobium meliloti* interaction the NF produced by *S. meliloti* induce a root hair curling, which is an early-plant response easy to follow. In the case of *Aeschynomene*, no NF are produced, and the rhizobial factors assumed to play an equivalent role do not induce root hair deformation. As a consequence, an early visible plant response is not available for *Aeschynomene* species. To bypass this difficulty, the p*MtENOD11::gusA-int* construct appeared as an appealing molecular marker of infection. ENOD11 symbiotic expression was observed in nodule primordia and later in a cell layer surrounding the infection zone but, unfortunately no early symbiotic expression of *ENOD11* could be detected

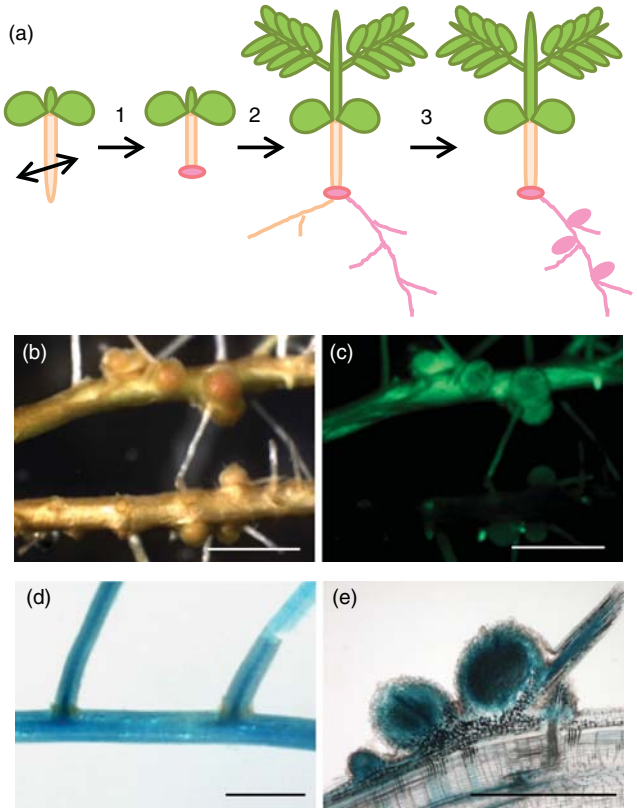

Figure 45.5 *Agrobacterium rhizogenes*-mediated transformation. (a) Steps to obtain transformed and nodulated roots: (1) hypocotyl cutoff and ARqua1 inoculation; (2) development of transformed roots; and (3) inoculation with *Bradyrhizobium* and subsequent development of nitrogen-fixing nodules. Transformed *A. evenia* nodules observed under either (b), white or (c), blue light for epifluorescence of green fluorescent protein (GFP), 35 days after transformation with *Agrobacterium rhizogenes* ARqua1 expressing the p*CaMV35S::GFP* construct. β-Glucuronidase staining in *A. evenia* transformed roots (d) and nodules (e) expressing the p*CaMV35S::gus-int* construct. Scale bars = 1 cm. (*Source*: Pictures reprinted from Arrighi et al. (2012) with permission.)

in *A. indica* (Bonaldi et al., 2010b). This illustrated the need for genomic resources to identify *Aeschynomene* genes differentially expressed during the establishment of the symbiosis that could be used as an early marker of infection in *A. evenia* for the development of bioassay strategies.

For this reason, *A. evenia* massive transcriptomic resources were established and used as a reference transcriptome for transcript profiling during symbiotic interactions (Cartieaux, unpublished data). The analyses of this data set will lead to the identification of candidate genes displaying a specific pattern of expression during the symbiotic interaction with the *Bradyrhizobium* partner. This library of putative new symbiotic genes associated with the ability of root transformation will pave the way for a successful gene candidate analysis in *A. evenia*.

45.2 CONCLUSIONS

The analysis of transcriptomic resources already constituted for *A. evenia* should rapidly allow us to highlight a subset of candidate genes specific to the Nod-independent symbiotic process, and the *Agrobacterium rhizogenes*-mediated transformation will permit to test their role via functional analyses. Moreover, the genetic dissection of the Nod-independent symbiosis, by screening a library of plant mutants altered in the symbiotic process, will constitute an essential approach that is complementary to the transcriptomic studies. It is such an approach that led to the identification of the key symbiotic genes in model legumes, and that could also bring to light specific determinants of the Nod-independent symbiotic infection process in *A. evenia*.

Although the absence of requirement of NF to trigger symbiosis has been identified to date in only 11 species of *Aeschynomene*, the mode of bacterial invasion and nodule organogenesis described in *Aeschynomene* spp. is largely shared by tropical legumes, including species of agronomic importance such as *Arachis hypogaea*. Peanut is the fourth largest oilseed crop in the world and is cultivated in more than 100 countries, with an annual production of 35.5 million tons (FAOSTAT [http://faostat.fao.org]). The phylogenetic proximity of *A. hypogaea* with *Aeschynomene* should allow knowledge transfer to this species with the goal of improving its nodulation efficiency. This work is a part of the valuation of tropical legumes, less studied than their temperate counterparts, while presenting an alternative symbiotic infection process. Understanding these mechanisms is a bottle neck in the current state of research on the ability to transfer the symbiotic nitrogen-fixation to nonlegume plants with the goal to improve agricultural production in a sustainable and environmentally way (Beatty and Good, 2011; Charpentier and Oldroyd, 2010; see also Chapters 5, 108, 109).

ACKNOWLEDGMENTS

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Chapter 46

Phosphorus Use Efficiency for N_2 Fixation in the Rhizobial Symbiosis with Legumes

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46.1 INTRODUCTION

Farmers in most developed countries are recommended to minimize nitrogen fertilization for environmental sake, whereas those in less developed countries cannot afford it in large agricultural areas. Thus, symbiotic nitrogen fixation (SNF) by legumes that already constitute a major input into agricultural and natural ecosystems may provide an ecologically acceptable complement or substitute for mineral nitrogen fertilizers. However, P deficiency is a major limiting factor for legume production where legume N nutrition largely depends on the rhizobial symbiosis, particularly in acidic and calcareous soils. Thus, low-soil P availability is a primary constraint to legume productivity in many low-input systems. It is also a limitation in high-input systems where soil chemistry converts the fertilizer P into less available forms, so that high P fertilization is inefficiently applied.

A possible explanation is that legumes have higher P requirements than nonsymbiotic plants (Vadez et al., 1996). Nevertheless, for common bean, it is possible to improve its SNF potential and expression under P deficiency (Pereira and Bliss, 1987; Vadez et al., 1999). However, the large genetic diversity of growth habits and duration, from bushy determinate to climbing indeterminate types, was not fully explored in the putative nine distinct gene pools of the species (Singh, 1991). In this study, we synthesize recent experiments that were performed in glasshouse hydroaeroponic culture in order to screen for genotypic diversity in P use efficiency (PUE) for SNF in *Phaseolus vulgaris*, *Vigna subterranea*, and *Medicago truncatula* and to search for candidate genes that may contribute to PUE for SNF. The nodulation and growth of the most contrasting recombinant inbred lines (RILs) were measured in field with multilocation participatory testing in various cultural systems of South France and North Africa in relation to soil P availability.

46.2 METHODS

46.2.1 Hydroaeroponic Culture of Nodulated Legume in Glasshouse

Legume seeds were surface-sterilized in 3% Ca hypochlorite for 30 min and subsequently washed with sterile distilled water. They were germinated in a Petri dish with 0.8% YEM Agar media. Selected uniform seedlings of each RIL were inoculated by soaking them 30 min into 100 ml broth containing around 10^8 cells ml^{-1} of one specific reference rhizobial strain as previously described for common bean (Hernandez and Drevon, 1991), cowpea (Alkama et al., 2009), and the *M. truncatula* model legume (Aydi et al., 2004).

Roots of inoculated seedlings were passed carefully through pierced rubber stopper, fixed with cotton fitted around the hypocotyl, and placed upon the topper of a $0.40 \times 0.20 \times 0.20$ m vat receiving 20 plants before transplanting into a temperature-controlled glasshouse. They were transferred during the third week into 1 l serum bottles for individual-plant culture until harvest during the sixth week when they had reached the flowering stage (R6-7). For microsymbiont assessment, nodulated seedlings were immediately placed into a 1 l serum bottle, just after germination and inoculation. The nutrient solution was intensely aerated with 400 ml compressed air per minute per liter solution and maintained around pH 7 through the addition of 1 g $CaCO_3$ per liter solution. The vats and bottles were distributed in a block design.

The symbiotic plants received 1 L $plant^{-1}$ of the nutritive solution from Hernandez and Drevon (1991), complemented with sterile distilled water and renewed every week. The solution contained 1 mmol urea starter N during the first 2 weeks and at transplanting into bottles. Thereafter, plants were grown with N-free nutrient solution. The starter urea concentration depended on legume spp. Every week, the deficient or sufficient P treatments were applied as KH_2PO_4

of which the amount in micromole plant^{-1} depended on legume spp.

46.2.2 Nodule *In Situ* Reverse Transcription and PCR Amplification

Three-millimeter-diameter nodules were harvested from 5-week-old plants in glasshouse and were thoroughly washed with diethyl pyrocarbonate (DEPC) treated water. They were then fixed overnight at 4 °C in tubes containing freshly prepared PFA (2% [v/v] paraformaldehyde, 45% [v/v] ethanol, and 5% [v/v] acetic acid).

Fixed-nodule sections were washed four times with DEPC-treated water under agitation during 5 min followed by 10 min twice to remove PFA. Thereafter, the nodules were included in low-melting agar 9% [m/v], dissolved with filtered phosphate-buffered saline (PBS) solution 1×, and cut into 50 µm sections. The nodule sections were transferred in tubes containing 0.5 ml of DEPC-treated water where the rest of agar was removed by three washings with DEPC-treated water at 60 °C. Then, the sections were added with 40 µl RT mix [8× buffer [5×], 1.25 dNTP [10 mM], 0.4 dig-11-dUTP, 3 Primer rev [10 µM], 6.7 DEPC-treated water], heated at 65 °C for 5 min, and then placed in ice for 2 min before the reverse transcriptase was added to each sample to a final concentration of 5 µmol l^{-1} before incubation at 42 °C for 1 h. Negative controls (NRT) were prepared by omitting the reverse transcription.

After reverse transcription, the RT mix was removed, and the samples were washed three times with 100 µl DEPC-treated water. Then, the PCR reaction was performed in 20 µl of DEPC-treated water and 20 µl of PCR mix [4× PCR buffer [10×], 1.2 $MgCl_2$ [50 mM], 0.8 dNTP [10 mM], 0.4 dig-11-dUTP, 1 Primer dir [10 µM], 1 Primer rev [10 cM], 0.2 Taq Poly [5 U/µl], 10.4 DEPC-treated water] with 30 cycles thermocycling [95 °C for 3 min; 95 °C for 30 s; 55 °C for 30 s; 72 °C for 45 s; 72 °C for 2 min] and at 20 °C for 5 min.

After amplification, the PCR mix was removed and the samples were washed three times with PBS 1×. Before detection of amplified cDNA, the samples were incubated in 100 µl of blocking solution (BSA 2% with triton 0.3% during 30 min under gentle agitation in darkness at 37 °C). Then the blocking buffer was removed and replaced by 100 µl of acid-phosphatase-conjugated anti-dioxygenin diluted 1 : 1000 in BSA [2%]. To remove anti-body excess, the samples were incubated during 90 min at room temperature and then washed during 5 min and 10 min twice with filtered PBS [1×].

Detection was carried out using the enzyme-labeled fluorescent ELF-97 endogenous phosphatase diluted 1 : 40 in the detection buffer, vigorously shaken, and filtered through a 0.22-µm filter. Samples were incubated in 20-µl ELF substrate–buffer solution for 20 min in the dark and transferred to washing buffer for three washings of 1 min, then mounted, and observed immediately with gray-scale view camera using ImageJ software as an image analysis program.

46.2.3 Nodular Diagnosis in Multilocation Participatory Field Assessment

From the screening in the glasshouse, two to six contrasting RILs were chosen for the multilocation field comparison. In each field, a homogeneous 20 by 10 m area was identified as a site. The RILs were sown in 2 m single-row within the local bean field in order to reduce border effects and to expose the RILs to similar environment and practices as the local bean cultivar. Up to 20 sites were chosen in each agroecosystem, constituting a randomized block design with each block being one farmer's site with RILs.

Like in glasshouse, nodulation and growth of the RILs were measured at the flowering stage when the first pod was 2 cm long for 50% of the plant population. Sampling was performed by excavating 20 cm in depth and around the root system of 10 plants per row, per block. Soil and plant samples were transferred in plastic bags and stored in a cold room until further analysis in laboratory.

The soil surrounding the roots was sorted from plants that were subsequently separated into shoot, root, and nodules. The plant parts were dried at 70 °C for 2 days. The available P of the soil was measured by the Olsen method according to standard NF ISO 11263 after extraction by soil agitation with a solution of sodium bicarbonate [0.5 M, pH 8.5].

Standard deviations were determined for all the traits. Analyses of variance and regressions were performed. Fixed effects in the analysis of variance were genotype and treatment or locations.

46.3 RESULTS AND DISCUSSION

46.3.1 Screening for N_2-dependent Growth in Low-P Controlled Environment

Following the initial screening with a common-bean core-collection (Vadez et al., 1999), the search for genotypes contrasting in PUE for SNF was performed in hydroaeroponics under glasshouse conditions with the population of the

cross between BAT477, tolerant to abiotic water-deficit, and DOR364, tolerant to biotic mosaic virus. A large diversity of adaptation to P deficiency was found with RILs 5, 6, 7, 12, 26, 28, 34, 60, 64, 75, 104, and 115 that were tolerant, in contrast with RILs 1, 4, 11, 24, 27, 29, 32, 37, 38, 62, 73, 83, 124, and 147 that were sensitive (Fig. 46.1). P deficiency decreased shoot/root ratio for all RILs (L'taief et al., 2012). Overall the RIL115 has been confirmed to be one of the most efficient genotypes with a mean N_2-dependent growth of 6 g DW $plant^{-1}$ under P deficiency, corresponding to a yield potential higher than 3 t grain ha^{-1}.

With similar methodology, RILs contrasting in PUE for SNF were found for *Vigna subterranea* (Fig. 46.2), *Vigna unguiculata* (Alkama et al., 2009), and *Medicago truncatula* (Mt) (Schulze et al., 2011). For these legume species, the value of optimal P was 75 and 30 µmol KH_2PO_4 pl^{-1} $week^{-1}$, respectively, whereas that for *P vulgaris* was 250 µmol KH_2PO_4 pl^{-1} $week^{-1}$.

Under controlled environment with starter N in hydroaeroponic culture, the growth of shoot could be expressed as a function of nodule growth as illustrated in Figure 46.3. The efficiency in use of the rhizobial symbiosis (EURS) is proposed as the slope of the linear part of the regression between plant growth and root nodulation. Thus, the EURS is the ratio of N_2-dependent growth/nodule mass. Indeed the growth depending on N_2 is the value of shoot biomass minus that corresponding to the growth of the plant without nodulation, that is, the growth exclusively due to the supply of N by the seed and the starter urea. The latter is estimated as the intercept of the curve with *Y* axis. Thus, the additional growth depended on the amount of N_2 fixed and the efficiency of its utilization.

Those genotypes having values above the mean EURS in Figure 46.3 were most efficient in PUE for SNF. They contrasted with those below the mean EURS that were the least adapted to P deficiency, especially those with low nodulation. Whatever the P supply, the mean EURS was higher for *M. truncatula* (Schulze et al., 2011) than, for *Vigna unguiculata* (Fig. 46.3) and for *Vigna subterranea*, meanwhile that for *Phaseolus vulgaris* being the lowest one (Drevon et al., 2011).

46.3.2 Physiological Characterization of Contrasting RILs

As illustrated in Figure 46.4, plants harbor various strategies to adapt to P deficiency. The external strategy links with the various processes involved with P acquisition, whereas the internal strategy links with distribution of P among plant organs and the efficiency of its utilization. For nodules, the internal strategy consists in optimizing the PUE for SNF.

In order to assess whether any phosphatase (APase) would be involved in the PUE for SNF, primers were designed from available sequences of candidate APase in the gene bank of *P. vulgaris* or other legume spp, including *Glycine max* and *M. truncatula*, for localization of the corresponding mRNA on cross nodule sections. The green signal in Figure 46.5a shows that phytase was expressed in nodules, and essentially localized in nodule cortex, both inner and middle. The higher increase in phytase signal (Lazali et al., 2013) and activity (Araújo et al., 2008) under P deficiency for the RIL115 compared to the less-efficient RIL147 suggests that the use of phytate in nodule cortex may contribute to P efficiency for the rhizobial symbiosis.

The expression of trehalose 6P APase in the outer-cortex of the nodule (Fig. 46.5b) might contribute to the control of various abiotic constraints by trehalose release (Bargaz et al., 2013), and that of phosphoenolpyruvate Apase (PEPase) (Fig. 46.5c) suggests inorganic P (Pi) mobilization from senescent cells (Bargaz et al., 2012). In the infected zone, both genes may supply Pi for bacteroidal metabolism. In the middle cortex, surrounding the internal cortex, the PEPase activation under P deficiency would not only supply additional Pi but also reduce active oxygen species through a nonenzymatic oxidative decarboxylation of pyruvate by hydrogen peroxide (Bargaz et al., 2012).

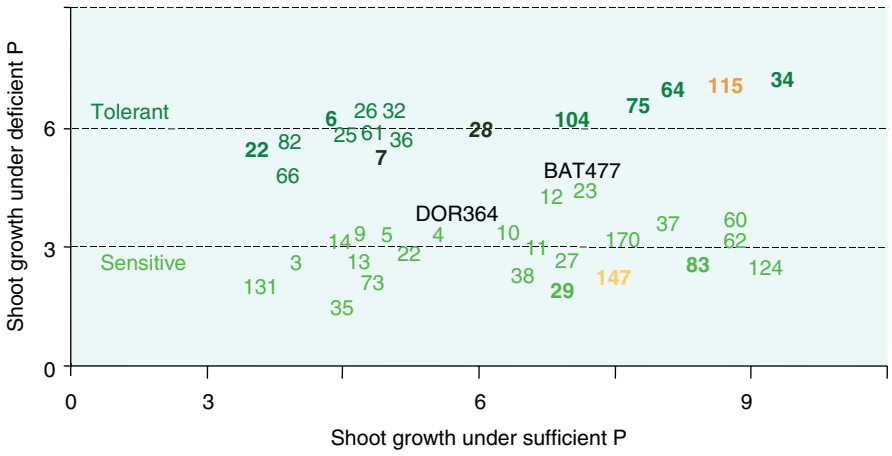

Figure 46.1 Shoot growth of recombinant inbred lines from the cross of BAT477 and DOR364 under sufficient versus deficient phosphorus supply. Data are mean of six replicates at least, harvested at flowering stage in hydroaeroponic culture with *Rhizobium tropici* CIAT899.

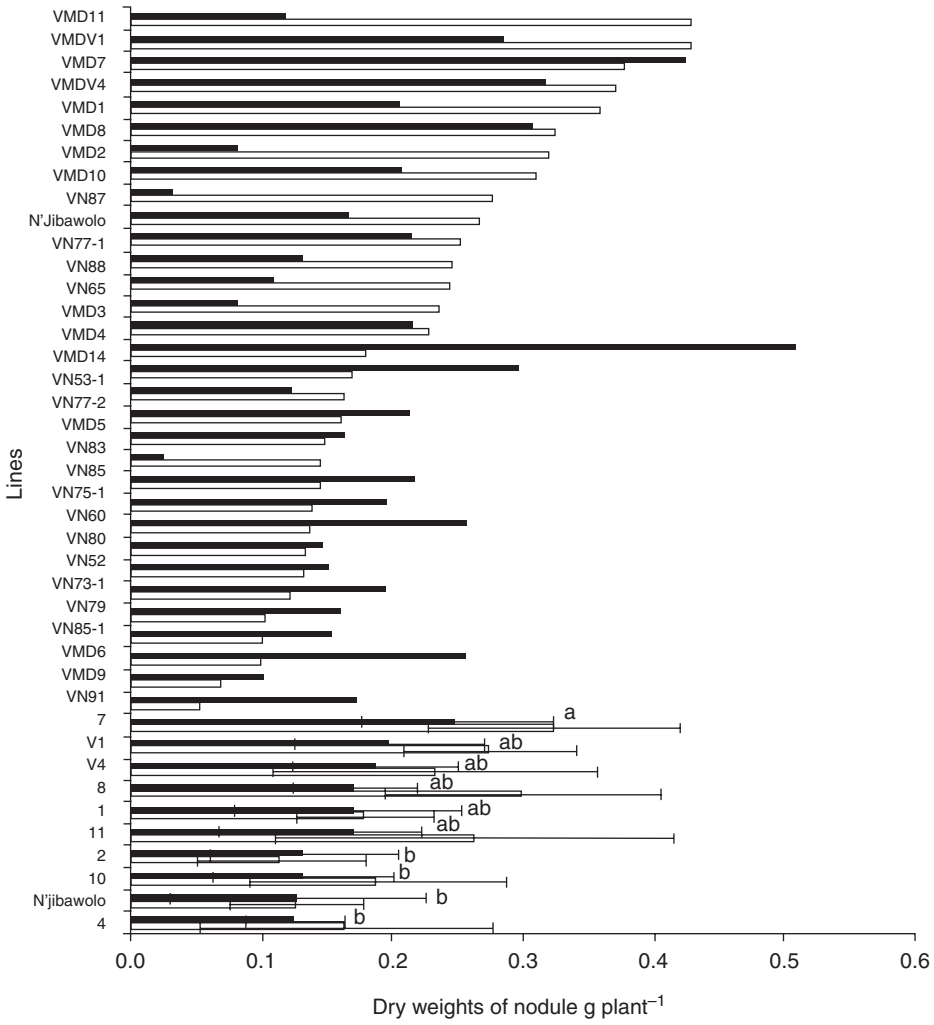

Figure 46.2 Nodulation of *Vigna subterranea* lines under sufficient versus deficient phosphorus supply. Data are mean and SD of six replicates, harvested at flowering stage in hydroaeroponic culture with *Rhizobium* sp. *Vigna* CB756.

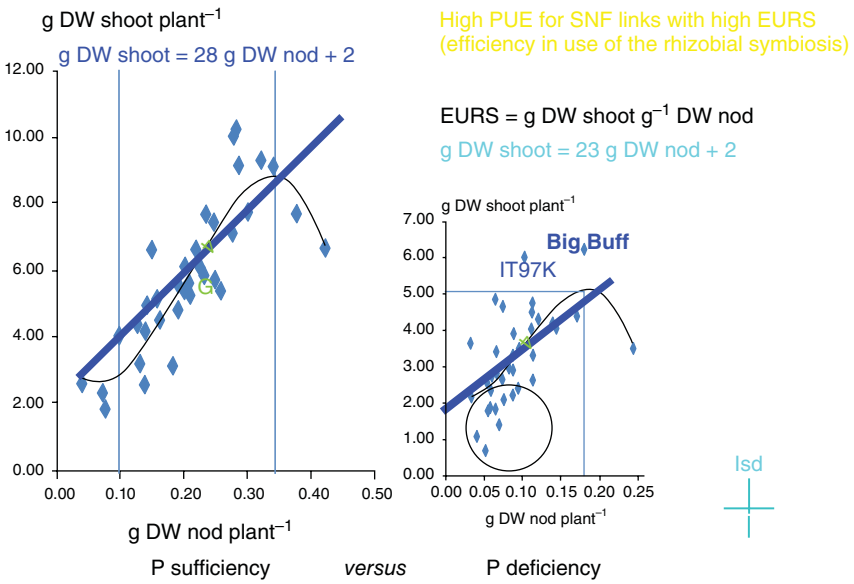

Figure 46.3 Relationship between nodulation and growth of *Vigna unguiculata* lines under sufficient versus deficient phosphorus supply. Data are mean of 10 replicates, harvested at flowering stage in hydroaeroponic culture with *Rhizobium* sp. *Vigna* CB756. (*Source:* From Hamdou.)

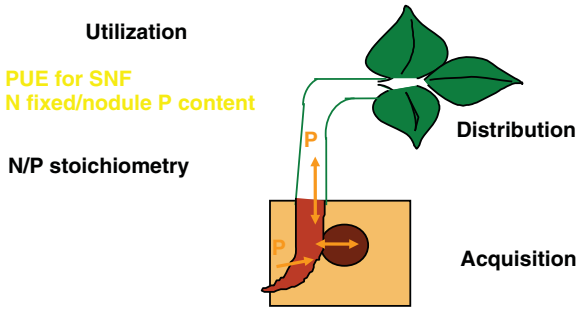

Figure 46.4 Strategies of nodulated legume to adapt to P deficiency.

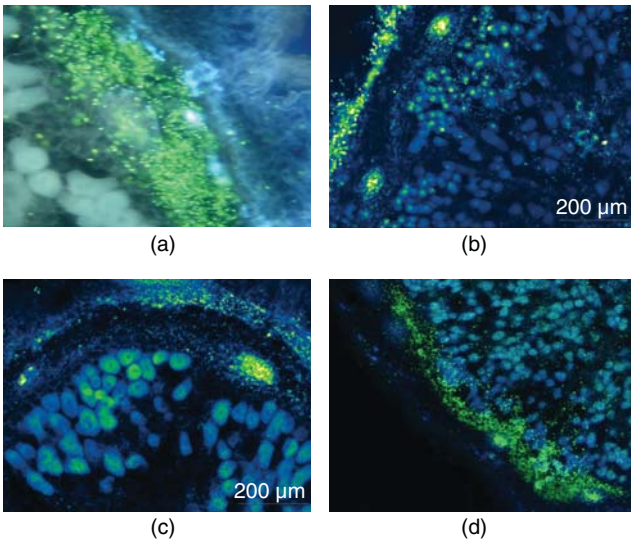

Figure 46.5 Transcript localization of phytase (a), phosphoenolpyruvate phosphatase (b), trehalose-6-phosphate phosphatase (c), and fructose-1,6-bisphosphate phosphatase (d) by *in situ* localization in nodule transversal section of the efficient common-bean RIL115.

All candidate genes of APases were intensely marked by fluorescence in the layers of inner cortex cells, that is, the tissue localized between vascular traces and the infected zone of nodule (Fig. 46.5). This reveals an intense metabolism of organic P in this tissue. It may supply Pi for the catabolism of sucrose from vascular traces into organic acids as the source of energy for bacteroids in the infected zone. Organic acids may also contribute as a source of energy and osmoticum for the turgescence of inner cortex cell that are presumed to be involved in the permeability of nodule cortex to gases (Serraj et al., 1995; Schulze and Drevon, 2005). Indeed this tissue has been suggested to be the oxygen barrier by regulating the entry of gases, in particular the O_2 that is involved in the respiratory synthesis of ATP for bacteroidal nitrogenase activity.

Thus, P deficiency was shown to increase the nodule conductance to O_2 diffusion, and the energetic cost of N_2 fixation under P deficiency in soybean (Ribet and Drevon, 1995)

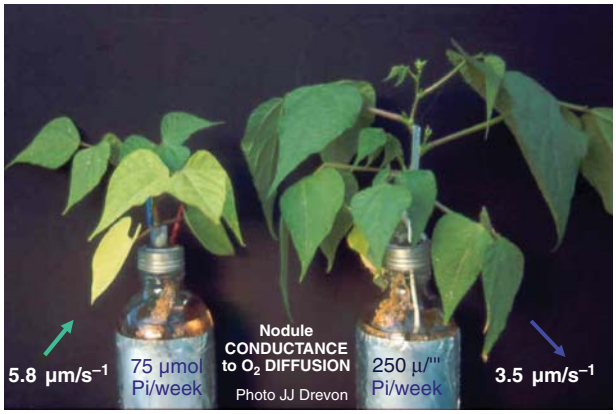

Figure 46.6 Effect of phosphorus deficiency on growth and nodule permeability to O_2 diffusion of common-bean inoculated with *R. tropici* CIAT899.

and *P. vulgaris* (Ribet and Drevon, 1995; Fig. 46.6) with the methodology described in detail in Jebara et al. (2005). This increase was concomitant with increase in gene expression of aquaporins (Fig. 46.7a) and carbonic anhydrase (Fig. 46.7b and c) in addition to APases. These results are consistent with the hypothesis of osmoregulation of the nodule conductance as illustrated in Figure 46.7d.

46.3.3 Field Observation of Contrasting RILs

The contrasting RILs 115 versus 147 were tested in fields in Lauragais (Southern France) during successive years with a participatory approach as described in Drevon et al. (2011). These RILs were also observed in organic horticultural farming of common bean in the Herault valley where, like in Lauragais, large variation in nodulation was found among sites as illustrated in Figure 46.8a. The simultaneous measurement of shoot and nodule biomass made it possible to establish the nodulation threshold for significant N_2 contribution to the growth of the plant, that is, the nodule mass below which no relation between growth of shoot and nodules was found. For the fields where the mean nodulation was above this threshold, a significant regression of shoot growth as a function of nodule mass was found (Fig. 46.8b), showing higher EURS for the efficient RIL115 than for the inefficient RIL147. This suggests that the selection for PUE and SNF may contribute to the improvement of the N_2-dependent growth of legumes in agro-ecosystems where low P in the soil is a limiting factor of SNF.

The contrasting RILs were also compared in agro-ecosystem of Kabylia. Four groups of soils could be identified from the analysis of physicochemical parameters: Group A for soils where N and P contents were high and nodulation was low, suggesting that nodulation was inhibited by high available mineral N that supported high plant growth

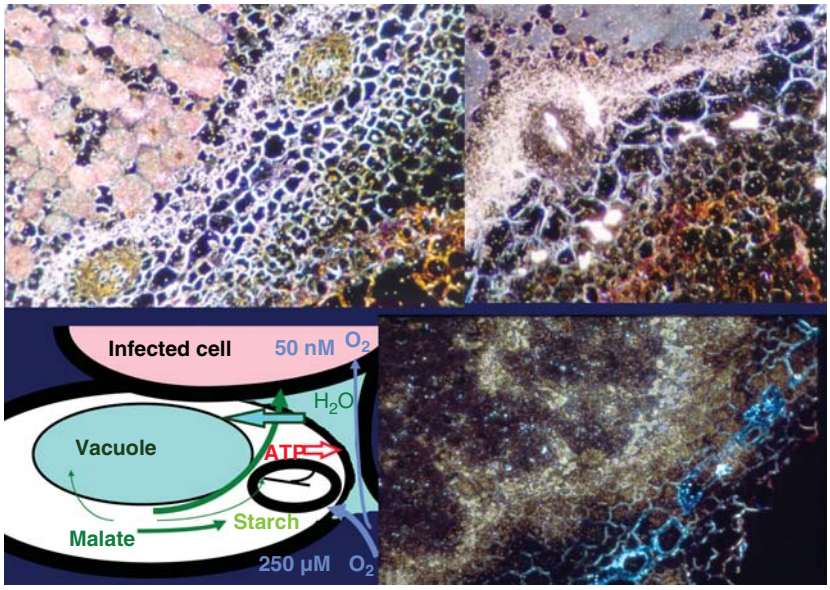

Figure 46.7 Transcript localization by *in situ* hybridization of aquaporin (a) and carbonic anhydrase under sufficient (b) versus deficient (c) phosphorus supply (Schump et al., 2003), and the hypothesis of osmoregulation of the nodule permeability (d) for the efficient common-bean RIL115.

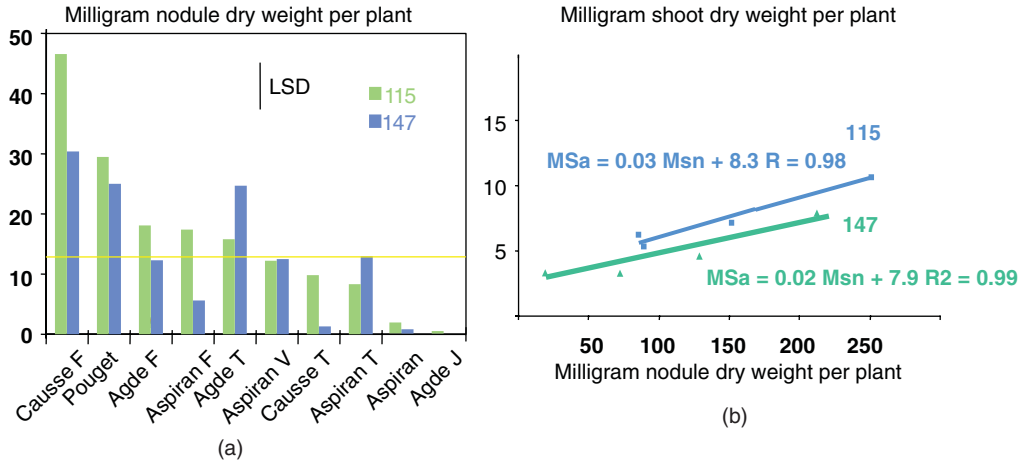

Figure 46.8 Spatial variation of nodulation (a) and the relationship with growth (b) of contrasting RILs 115 and 147 of *P. vulgaris* in Hérault valley in 2006.

without N_2-dependance; Group B for soils where N content and nodulation were low, although soil P content was not particularly low, suggesting that the low nodulation might be attributed to low population of native rhizobia and that rhizobial inoculation could be tested; Groups C for soils with relatively low P and N contents and low plant growth and nodulation, though nodulation was higher than in groups A and B, suggesting that low nodulation might be due to low soil P content; Group D for soils with higher P content than Group C, where nodulation and growth were the highest with relatively low soil N content. For C and D soil groups, a relationship between mean nodulation and Olsen P contents was found (Fig. 46.9).

46.3.4 Interactions with Soil Microorganisms

In order to assess whether the rhizobia may influence the use of P for nitrogen fixation, cross-inoculation trials were performed with rhizobia isolated from agro-ecosystems. Thus, some rhizobia were found to compensate the low PUE for SNF of such genotypes as RIL124 (Fig. 46.10). This suggests that the genotype of not only the macrosymbiont, that is, plant, but also the microsymbiont, that is, rhizobia, may contribute to the PUE for SNF.

In order to assess whether rhizobial phosphatase could be involved in this contribution, primers were designed for

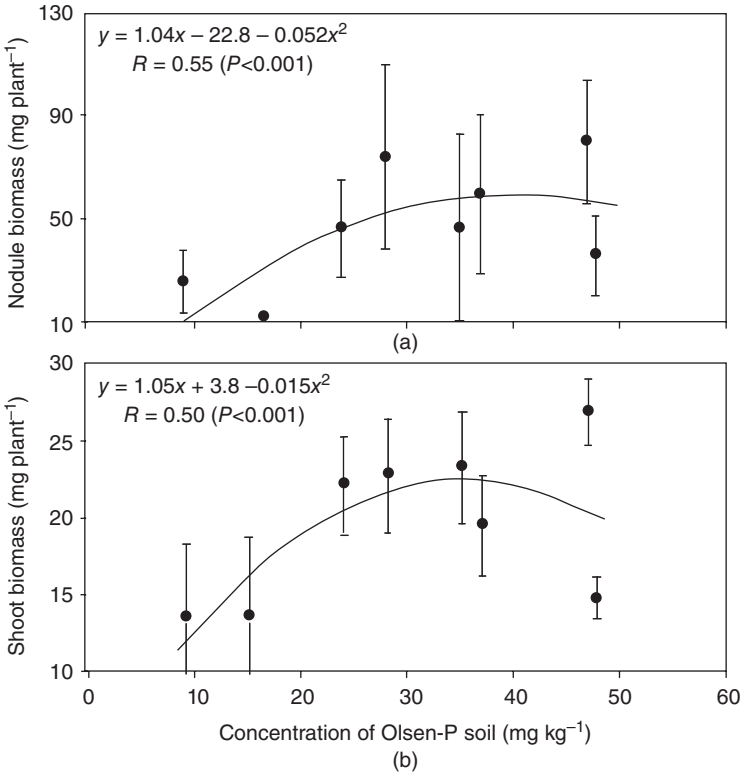

Figure 46.9 Nodule (a) and shoot (b) biomasses, versus concentration of Olsen P in soil, for El Djadida (Dj) and the RILs 147, 124, 115, 104, 83, 75, and 7 through multilocation test in Kabylia (Algeria). Data are means and SD of 10 replicates collected at flowering stage for each line and site.

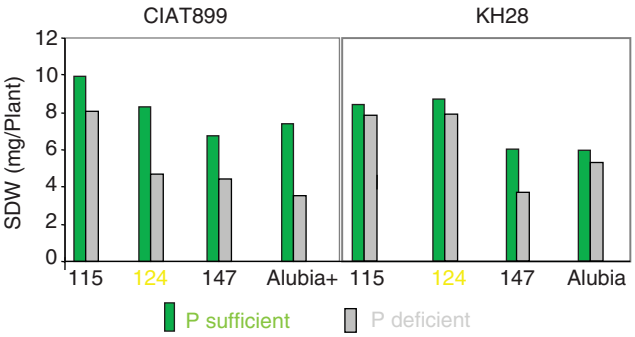

Figure 46.10 Native rhizobia KH28 increases the tolerance to P deficiency for common-bean RIL124 and Alubia (Zaman-Allah et al., 2006).

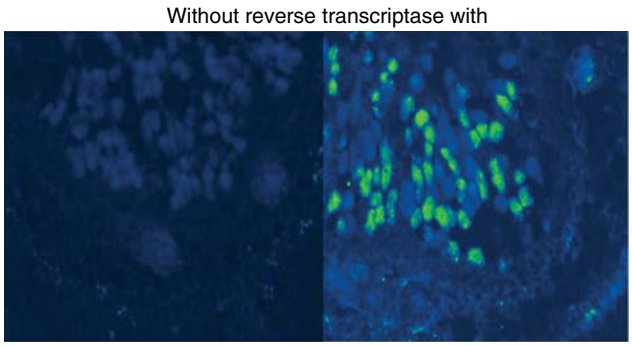

Figure 46.11 Localization of transcripts (green fluorescent signal) of rhizobia acid phosphatase in nodule transverse sections of the efficient common-bean RIL115 (left) versus the deficient common-bean RIL147 (right) as a function of P treatments. P deficiency (left) versus P sufficiency (right) as 75 versus 250 µmol Pi $week^{-1}$ pl^{-1}.

bacterial APase from sequences of whole genome of various rhizobia. The green signal in Figure 46.11 shows that the acid phosphatase was expressed in some infected cells of the nodule infected zone.

As the mycorrhizal partner may influence the PUE for SNF, the tripartite symbiosis was examined in hydroaeroponic culture after preculture in sandlike, described in details in Tajini et al. (2009). The effect of mycorrhizal infection on nodulation varied according to species, *Glomus intraradices* being the most efficient whereas *Gigaspora* sp. was detrimental whatever the RILs.

In order to assess whether the variation in PUE for SNF might alter the soil P availability, the Olsen P was measured in the rhizosphere soil separated from the nodulated roots during the nodular diagnosis. Some fields of Herault valley were found with higher P availability in the soil interacting with nodulated roots of RIL115 than in that of RIL147 (Fig. 46.12). This suggests that the high PUE for SNF of RIL115 has stimulated the mechanisms associated with root for transformation of soil organic P and mineral P into soluble Pi as the only source of P that the roots can absorb. Whether those mechanisms would be due to rhizospheric microorganisms, or exclusively due to root excretions, remains to be established in our experiments.

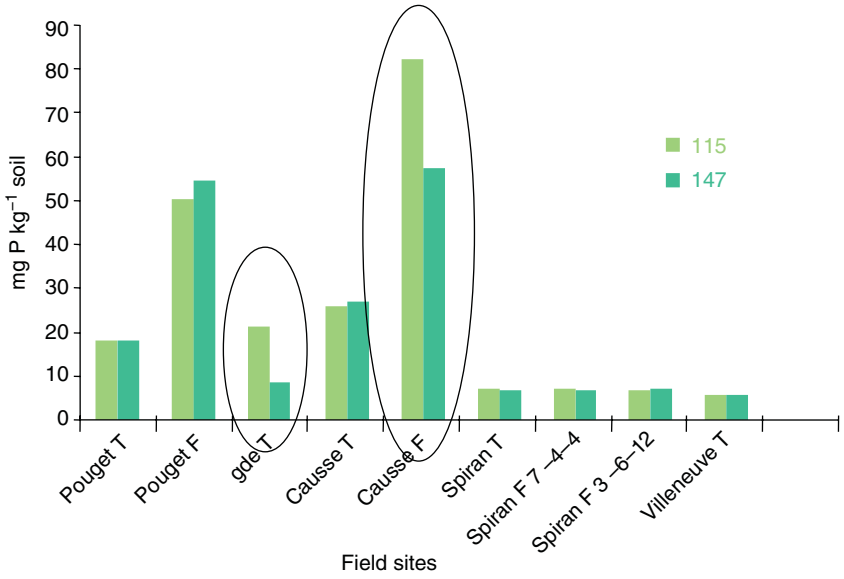

Figure 46.12 Differences in Olsen P among field sites and RIL rhizosphere in Herault valley.

In other fields (Fig. 46.12), Olsen P was comparable for both RILs, suggesting that some soil microbes may have compensated the effects of low PUE on soil P availability for RIL147. Therefore, P-solubilizing rhizospheric bacteria or fungi could be isolated preferentially from those soils in order to test subsequently in hydroaeroponics whether they do contribute to the increase in Olsen P.

46.4 CONCLUSION

Genotypic variations in PUE for SNF could be found in hydroaeroponics among various legume species, including so far *P. vulgaris*, *V. unguiculata*, *V. subterranea*, and the model legume *M. truncatula*. Therefore, this parameter may be suggested to legume breeding in order to ensure that selected legumes have high potential for N_2 fixation and efficient use of P. Thus, the phosphorus is becoming a scarce, nonrenewable resource. Our physiological work suggests that some APases may contribute to P efficiency for SNF in nodules. This is the case of the plant phytase that we recently discovered in common bean nodule. More studies are therefore needed on the diversity of plant phosphatases in nodules and their contribution to the N_2 fixation process and the adaptation of nodulated legumes to P deficiency. Among the mechanisms involved in this adaptation is the regulation of the nodule permeability to O_2 with the following hypothesis of osmoregulation of the FSN: The energy metabolism determining the symbio-rhizobial nitrogen fixation was regulated by the variations of the cellular volume of the inner nodular cortex (parenchyma surrounding the infected zone), which changed the nodular permeability modifying the twisting of the intercellular spaces of the cortex through which O_2 diffuses (Fig. 46.5). The variations in cellular volume would be determined by the internal variations in nitrogen demand of the plant. They could also be affected by the variations in physicochemical environment of the nodules.

The multilocation tests show that the genotypes with high PUE for SNF do better adapt to low-P soils. This confirms that the *in vitro* selection of high PUE and SNF in hydroaeroponics is a methodology to breed for legumes with potentially better adaptation to low-P soils. However, the tests also show some fields where less-efficient genotype may adapt probably because of beneficial interactions with soil microbes. Thus, the fields where the nodulation of the least efficient symbiosis was as high as that of the most efficient symbiosis, as illustrated in Figure 46.8, may be the source of efficient microbes to compensate for low P efficiency of the plant. Therefore, nodules and roots of the plants sampled on these soils have been preserved in order to identify their rhizobia, mycorrhiza, and rhizobacteria.

These microbes will be subsequently tested in hydroaeroponics in order to assess whether they do contribute to the adaptation to P deficiency and whether they harbor candidate phosphatase genes for adaptation to low-P soils. If they would confirm such potential, then these microbes could be considered for inoculation tests in those fields where the genotypes with low PUE for SNF did perform weakly. Such an approach could lead to precision inoculation, consisting of transferring beneficial local microbes to the fields where the requirement for inoculation has been established by the nodular diagnosis.

The earlier assessment of the contribution of soil microorganisms for P efficiency is based on the comparison in hydroaeroponics of their effect on the N_2-dependent growth of genotypes contrasting in PUE for SNF. This environment makes it possible to perform *in situ* open-flow gas

exchange measurements to assess the energetic metabolism supporting SNF as physiological basis of the efficiency. The localization and intensity of the expression of candidate microbial genes for P efficiency is possible by *in situ* RT-PCR, on the basis of our ongoing observation with phytases of rhizobia and *Bacillus subtilis*.

Overall, the earlier observations lead us to postulate the following hypothesis of a virtuous cycle of fertility due to be symbiotic and rhizospheric microorganisms associated with legumes: because legumes require P for their SNF, they would have developed intensive interactions with soil microorganisms for acquiring P from soil and the benefit of this cycle for soil fertility would increase with raising PUE for SNF.

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Chapter 47

Regulation of Nodule Development by Short- and Long-Distance Auxin Transport Control

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47.1 INTRODUCTION

Nodules are root organs induced by rhizobia on legume roots, following the perception of specific Nod factors by the host. Similarly, actinorhizal plants form nodules in symbiosis with *Frankia* bacteria. It has been argued that because nodulation emerged recently during evolution, approximately 60 million years ago (Sprent, 2007; 2008), it is likely that the mechanisms that regulate nodule development were co-opted from existing developmental processes, most likely those that regulate lateral root development (Hirsch and LaRue, 1997), a process that has existed for approximately 400 million years (Raven and Edwards, 2001).

Nodules and lateral roots are formed post-embryonically from endogenous cell types that are triggered to divide, then form an organ primordium, and later differentiate and elongate. In the case of lateral roots, cell divisions first occur in the pericycle, a lateral root primordium is formed after further divisions, and the primordium differentiates into an organ with a central stele. An apical meristem becomes active and leads to lateral root elongation. Depending on the host species, nodule development can be based on modifications of existing lateral or adventitious roots or involve *de novo* induction of cell divisions in pericycle and cortical cells. Nodule development in the nonlegumes shows similarities to lateral root formation. In actinorhizal plants, *Frankia* first cause cortical cell divisions to form a pre-nodule that is colonized by hyphae, and then trigger pericycle cell divisions to form a lateral root-like nodule. *Frankia* invade the cortical cells of this nodule, which retains a central stele, similar to lateral roots (Pawlowski and Bisseling, 1996; see Chapters 42, 43, 55). In *Parasponia*, the only nonlegume known to

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nodulate with rhizobia, the symbionts trigger the initiation of a lateral root that they later invade (Trinick, 1979; see Chapter 4). Nodule structures in legumes are diverse, as discussed later, and typically involve the initiation of a nodule from pericycle and/or cortical cells *de novo*, resulting in a nodule with peripheral vascular strands (Hirsch, 1992).

Two different nodule types have been studied in detail. Temperate legumes, for example, pea, clover, alfalfa, and the model legume *Medicago truncatula*, usually form indeterminate nodules, which are characterized by nodule initiation in the inner cortex and also usually the pericycle (Timmers et al., 1999). The resulting nodules form a persistent nodule meristem, which allows continuous growth and leads to the formation of elongated nodules. Determinate nodules are formed on many (sub)tropical species, including soybean, bean, and the model legume *Lotus japonicus*, and are initiated in the outer root cortex by cell enlargement and divisions. Cell divisions are later induced in the pericycle and inner cortex, and both cell division sites subsequently merge. These nodules are typically spherical because the nodule meristem differentiates and disappears (Rolfe and Gresshoff, 1988).

There is evidence that auxin is synthesized by the nitrogen-fixing symbionts themselves, including the actinomycete *Frankia* (Wheeler et al., 1984), *Azospirillum* (see Chapters 90, 91) and rhizobia (Kefford et al., 1960). More importantly, the symbiont indirectly alters auxin transport and localization inside the host root (Mathesius, 2008). For a general review on the role of hormones in nodulation (see Chapter 56).

If nodule development has been modified from the process of lateral root formation, it is likely that similar developmental signals regulate both processes. A key regulator of lateral root development is auxin (Casimiro et al., 2003; Fukaki et al., 2007). Auxin patterns in the plant are crucial for determining subsequent developmental patterns (Heisler et al., 2005). Auxin is not only a pattern-determining regulator but also controls cell division, cell elongation, and vascular tissue differentiation (Teale et al., 2006; Woodward and Bartel, 2005). It has long been suggested that auxin is also a regulator of nodule development (Hirsch, 1992; Hirsch and Fang, 1994; Thimann, 1936). There are several possible mechanisms by which rhizobia or *Frankia* symbionts could initiate nodule development through the involvement of auxin in the host, for example, through alteration of auxin synthesis, breakdown, signaling, or transport in the host (Mathesius 2008).

47.1.1 Regulation of Auxin Transport in the Plant

Auxin is thought to be mainly synthesized in young shoot tissues and transported from there to other tissues and organs, although many other tissues can also synthesize auxin (Ljung et al., 2002). Auxin can occur as the free active form, primarily indole acetic acid (IAA), or it can be conjugated for storage. Tracking of radiolabeled auxin indicates that transport of auxin from the shoot to the root tip occurs through the vascular tissue, while transport in the root from its tip to the elongation zone occurs through epidermal cells (Mitchell and Davies, 1975; Tsurumi and Ohwaki, 1978). In addition to long-distance auxin transport, local transport of auxin along and across tissues is important for auxin localization in small groups of cells, for example, in an emerging lateral root (Grieneisen et al., 2007; Jones, 1998). Although auxin can be transported within the plant via the phloem from source to sink tissues, polar auxin transport can be regulated specifically by polar-active transport (PAT) through auxin transport proteins.

Auxin is a weak acid; when present in the acidic cell wall environment, it occurs as the protonated form (IAAH) and can enter cells partially by diffusion. It can also be actively transported into cells by auxin importers of the amino acid permease families AUX1 (auxin resistant 1), LAX (like-AUX1), and PGP4, a member of the MDR/PGP (multidrug resistance/P-glycoprotein) families (Terasaka et al., 2005; Yang et al., 2006). AUX1 has been suggested to play a role in auxin unloading from the phloem and loading into the PAT system (Swarup et al., 2001).

Due to the higher pH inside the cytoplasm, deprotonated auxin (IAA^-) cannot diffuse out of a plant cell but requires active export by transporters of the PIN (Pin-formed) and PGP families (Geisler et al., 2005; Petrasek et al., 2006). The polarity of auxin transport is established by the polar localization of PIN proteins on either the basal or apical side of the cell (Wisniewska et al., 2006). Different members of the PIN family are localized in a cell- and developmental-specific pattern, for example, PIN1 is localized on the apical side of vascular cells in the root and mediates acropetal auxin flow, whereas PIN2 is localized at the basal side of epidermal cells in the root tip where it mediates basipetal auxin flow. Mutations or misexpression of *PIN* genes have been shown to cause changes in local auxin accumulation and plant development (Friml, 2003; Vieten et al., 2007).

Auxin export can be inhibited by synthetic and natural auxin efflux inhibitors, including NPA (1-*N*-naphthylphthalamic acid) and TIBA (2,3,5-triiodobenzoic acid), which bind to the so-called NPA-binding proteins (NBPs). The NBPs have been suggested to interfere with PIN activity through a possible third protein (Muday and DeLong, 2001). AEIs (auxin export inhibitors) also affect auxin transport by inhibiting actin dynamics, which are required for PIN cycling (Dhonukshe et al., 2008). In addition, NPA inhibits auxin export by binding to MDR/PGPs (Geisler et al., 2005; Murphy et al., 2002; Noh et al., 2001). Flavonoids are a class of natural AEIs, some of which have been shown to regulate

PIN activity and localization in *Arabidopsis* (Peer and Murphy, 2007). Flavonoids with specific structures, especially flavonols, have been found to inhibit auxin transport by competing with synthetic AEIs for plasma membrane and microsomal binding sites (Bernasconi, 1996; Jacobs and Rubery, 1988; Stenlid, 1976). Flavonoids are likely to have several targets in plant cells, as they have been shown to interact with PGP auxin transport proteins (Bernasconi, 1996) as well as with an aminopeptidase (Murphy and Taiz, 1999). The flavonol quercetin enhanced auxin uptake by PGP4 in a heterologous system (Terasaka et al., 2005) and reduced auxin export by PGP1 in a manner similar to that of NPA (Geisler et al., 2005). In *Arabidopsis*, the lack of flavonoids alters the expression and localization of certain PIN proteins, and it has been suggested that flavonoids could act by targeting PIN intracellular cycling (Peer et al., 2004). Auxin has also been shown to increase *PIN* gene expression in a positive-feedback loop (Vieten et al., 2005). Studies in flavonoid-deficient *Arabidopsis* mutants confirm that these plants have higher rates of auxin transport, whereas mutants overaccumulating flavonols show decreased auxin transport rates (Brown et al., 2001; Murphy et al., 2000; Peer et al., 2004). Flavonoids are likely to link auxin transport with changes in the environment because flavonoids are accumulated in response to a variety of biotic and abiotic stimuli (Buer and Muday, 2004; Taylor and Grotewold, 2005). The colocalization of flavonoids at sites of high auxin concentration supports their role in auxin transport control (Buer and Muday, 2004; Buer et al., 2006; Murphy et al., 2000; Peer et al., 2001).

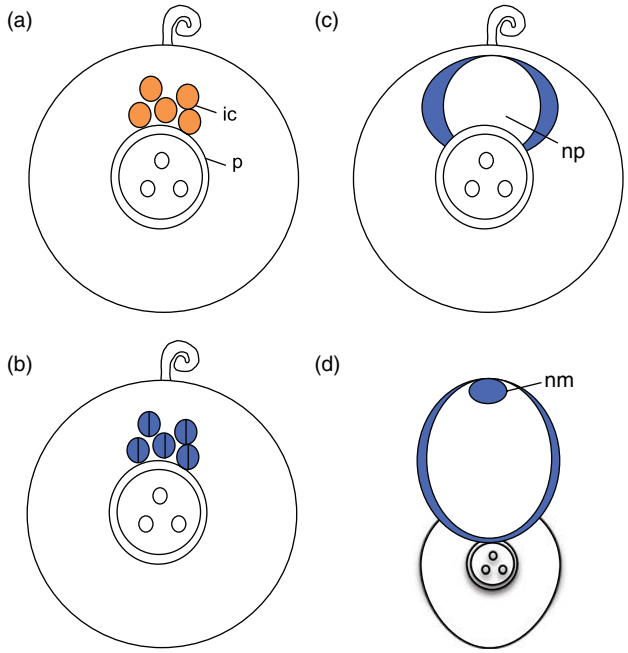

Figure 47.1 Schematic diagram showing areas of auxin accumulation in indeterminate nodule formation. (a) Flavonoid accumulation (orange) in nodule precursor cells preceding cell division. (b) Auxin accumulation (blue) in early dividing cortical cells. (c) Auxin accumulation in young nodule primordia becomes restricted to the nodule periphery. (d) Auxin accumulation in mature nodules is restricted to vascular tissue at the nodule periphery and the nodule meristem. ic = inner cortex; p = pericycle; np = nodule primordium; and nm = nodule meristem. The curled root hair at the top of each section indicates the inoculation site.

47.1.2 Rhizobia Alter Auxin Responses During Nodule Development

Auxin was first connected with the formation of nodule legumes with the discovery of increased auxin concentrations in mature nodules (Thimann, 1936). Since then, auxin response genes, for example, *DR5* and *GH3*, have been used to map the detailed auxin responses, presumably reflecting auxin distribution, during nodule development (Fig. 47.1). In white clover, which forms indeterminate nodules, rhizobia causes a reduction in *GH3* activation at and below the site of infection within 10 h after infection (Mathesius et al., 1998b). This is followed by an increase in expression at the site of nodule initiation 24 h after inoculation. Similarly, *DR5* expression appears to be interrupted below the site of nodule initiation in *M. truncatula*, but increases in a developing nodule (Huo et al., 2006). *GH3:GUS* expression has been observed in the early dividing pericycle and cortical cells of a forming nodule in white clover (Mathesius et al., 1998b) and in *M. truncatula* (van Noorden et al., 2007). *GH3:GUS* expression is high in the early nodule primordium of white clover, but low in the center of a differentiating nodule, remaining only in the nodule meristem and the vascular bundles (Mathesius et al., 1998b). Similarly, high *GH3:GUS* expression has been found in the dividing outer cortex cells in *L. japonicus* (Pacios-Bras et al., 2003; Takanashi et al., 2011), and more recently a sensitive *DR5:GFP* reporter was used to localize auxin responses in the earliest dividing cortical cells in *L. japonicus* (Suzaki et al., 2012). During later stages of determinate nodule development, *GH3:GUS* and *DR5:GFP* expressions are present in peripheral vascular tissue and meristematic cells (Pacios-Bras et al., 2003; Suzaki et al., 2012; Takanashi et al., 2011). Interestingly, *DR5:GUS* expression is altered in *M. truncatula* mutants with altered vascular bundle formation, confirming the idea that auxin localization is important for vascular bundle differentiation in developing nodules (Guan et al., 2013).

The use of nodulation mutants has shown that the auxin responses in early nodule primordia are always associated with the ability to form cell divisions. For example, in spontaneous nodule-forming mutants of *L. japonicus*, auxin responses have been found in the cortex, whereas in nonnodulating mutants no such responses were seen (Suzaki et al., 2012). Computer modeling of the auxin maximum

formed in inner or outer cortical cells of indeterminate and determinate nodule types has been used to investigate whether experimentally observed auxin responses are likely to result from increased auxin synthesis, reduced auxin export, or increased auxin import into the cortical cells. The study concluded that the broad auxin maximum seen in the cortex during nodule initiation is most likely due to decreased auxin efflux from these cells (Deinum et al., 2012; see Chapter 66). In addition, the changes in auxin accumulation in the inner and outer cortex, observed in indeterminate and determinate legumes, respectively, could be explained by slight lateral shifts in auxin exporter localization.

47.1.3 Regulation of Auxin Transport Preceding Nodule Initiation

The observed changes in auxin response, most likely reflecting auxin accumulation, during nodule development are regulated by the plant upon Nod-factor perception and are most likely due to changes in auxin transport. In several legumes, it has been observed that synthetic auxin transport inhibitors can induce nodules spontaneously in the absence of rhizobia (Allen et al., 1953; Rightmyer and Long, 2011; Wu et al., 1996), and this is accompanied by similar expression of certain nodulation genes as in normal nodules (Hirsch et al., 1989). In addition, the reduction of *GH3* expression observed during the early stages of indeterminate nodule formation can be mimicked by Nod factors and the synthetic auxin transport inhibitor NPA (Mathesius et al., 1998b). These findings suggest that rhizobia inhibit auxin transport in legumes forming indeterminate nodules before the onset of cell divisions. This is supported by measurements of radiolabeled auxin transport in roots of *Vicia sativa*, which shows that rhizobia, and specifically functional Nod factors, inhibit polar auxin transport within 24 h of inoculation (Boot et al., 1999). Similarly, a local inhibition of auxin transport has been found in *M. truncatula* following spot-inoculation of the susceptible root zone (van Noorden et al., 2006; Wasson et al., 2006). However, no inhibition of auxin transport was detectable in the legume *L. japonicus*, which forms determinate nodules, before nodule initiation (Pacios-Bras et al., 2003). Known regulators of auxin transport are flavonoids (Jacobs and Rubery, 1988), ethylene (Burg and Burg, 1966), and cytokinin (Laplaze et al., 2007). Rhizobia induce the accumulation of flavonoids (Mathesius et al., 1998a), ethylene (Ligero et al., 1987), and activate cytokinin signaling (Plet et al., 2011) during nodulation.

Flavonoids are induced in the cortical and pericycle precursor cells of a nodule after inoculation of nodulating rhizobia or Nod-factor treatment (Mathesius et al., 1998a). Flavonoids also accumulate in the cortex after treatment of white clover roots with cytokinin, which induces cortical cell divisions (Mathesius et al., 2000). To test if flavonoids are required for auxin transport inhibition by rhizobia, the first enzyme of the flavonoid biosynthetic pathway, chalcone synthase, was silenced using RNA interference in *M. truncatula* (Wasson et al., 2006). The resulting flavonoid-deficient roots do not nodulate and auxin transport inhibition by rhizobia is not observed, confirming that flavonoids are necessary for nodulation and for auxin transport inhibition during indeterminate nodule development in *M. truncatula* (Fig. 47.2). Silencing of sub-branches of the flavonoid pathway in *M. truncatula* suggests that it is most likely the flavonols that are responsible for auxin transport regulation (Zhang et al., 2009). The flavonol kaempferol is upregulated following rhizobia inoculation, and exogenous application of kaempferol is able to restore auxin transport control in flavonoid-deficient roots and rescue nodulation (Zhang et al., 2009). Silencing of the isoflavonoid pathway in soybean has shown that isoflavonoids are crucial for nodulation as Nod gene inducers, but are unlikely to be required for auxin transport regulation in the development of a determinate nodule (Subramanian et al., 2006). As auxin transport inhibition is not detectable after rhizobia inoculation in soybean (Subramanian et al., 2006) or *L. japonicus* (Pacios-Bras et al., 2003), it is possible that the inhibition of auxin transport is unique to legumes forming indeterminate nodules (Subramanian et al., 2007; Wasson et al., 2006).

Ethylene acts as a negative regulator of nodulation (Guinel and Geil, 2002). The effect of ethylene is local, that is, ethylene acts in the root, as established by grafting experiments (Prayitno et al., 2006). The ethylene-insensitive *sickle* mutant of *M. truncatula*, which hypernodulates (Penmetsa and Cook, 1997), still shows auxin transport inhibition at the root tip following inoculation with rhizobia (Prayitno et al., 2006), consistent with the requirement of auxin transport inhibition for nodule initiation. Within 24 h, an increase in auxin transport observed in wild-type roots is exaggerated in *skl*, and this was accompanied by an increased expression of *PIN2* and an increased number of nodules initiated at the site (Prayitno et al., 2006). This suggests that ethylene synthesis and/or perception could downregulate the auxin accumulation at the site of nodule initiation (Fig. 47.2).

Cytokinin is known to interfere with auxin accumulation during lateral root initiation by altering PIN gene expression (Laplaze et al., 2007). In the cytokinin-insensitive and non-nodulating *M. truncatula* mutant *cre1*, auxin transport is elevated and auxin transport inhibition by rhizobia is abolished (Plet et al., 2011). This is accompanied with altered *PIN* gene expression and localization, suggesting that cytokinin signaling acts upstream of auxin transport regulation. This is supported by results from *L. japonicus*, where *DR5:GUS* expression was observed in spontaneous nodules in the *snf2* mutant, which shows constitutive cytokinin signaling (Suzaki et al., 2013; Suzaki et al., 2012). In addition, other Nod-factor signaling components act upstream of auxin transport regulation because synthetic auxin transport

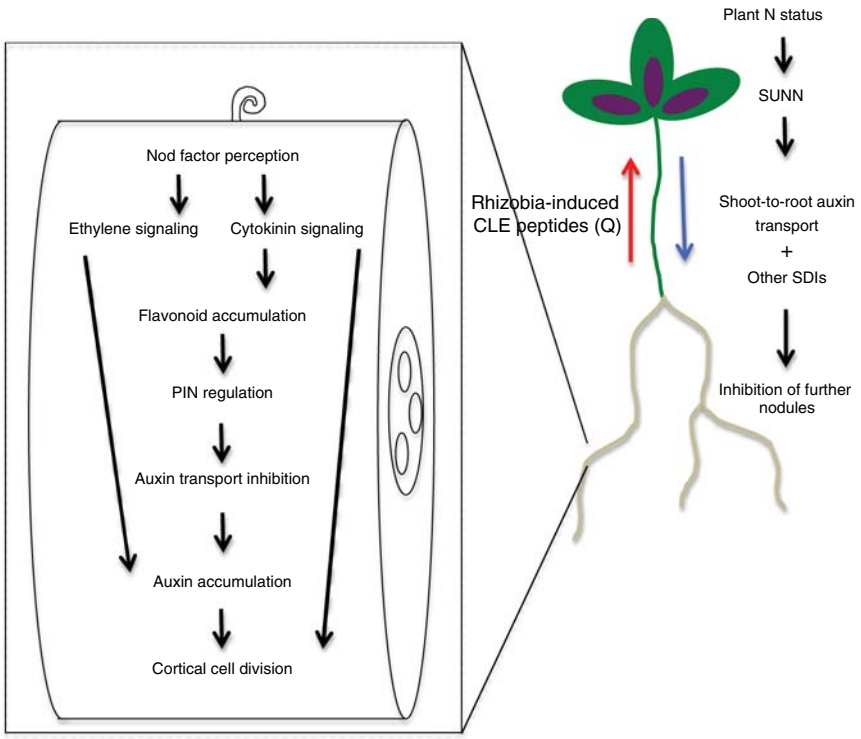

Figure 47.2 Simplified diagram showing the major known steps in local and long-distance auxin transport regulation in indeterminate nodule types. Upon inoculation with rhizobia (curled root hair) and early Nod-factor signaling, both ethylene and cytokinin signalings are activated. Ethylene signaling has been shown to affect auxin accumulation by increasing local auxin transport. Cytokinin signaling most likely acts upstream of auxin transport changes and also most likely upstream of the accumulation of flavonoids, which are hypothesized to regulate auxin transport via changes to PIN protein activity. Following the transient inhibition of auxin export at the inoculation site, auxin accumulates in inner cortical cells, and this is associated with the division of these cortical cells. At a so far undefined step during this local induction of nodulation responses, a signal is sent to the shoot, most likely a CLE-related peptide, which directly or indirectly activates autoregulation via NARK (SUNN in *M. truncatula*). This, together with the plant N status, controls the shoot-to-root auxin transport that is associated with further inhibition of nodulation. Other shoot-derived inhibitors (SDIs) are also likely to be involved in this autoregulation.

inhibitors are able to induce pseudonodules in a range of early nodulation mutants including *nfp, lyk3, dmi1, dmi2, dmi3, nin,* and *rit1,* but not in the ethylene-insensitive mutant *skl* (Rightmyer and Long, 2011).

47.1.4 The Role of Auxin in Nodule Initiation and Differentiation

Auxin transport inhibition preceding nodule initiation is followed by increased auxin transport and increased auxin responses at the site of nodule initiation (Mathesius et al., 1998b). Increased auxin concentrations are also found within 24 h of inoculation in bean (Fedorova et al., 2000), and strong induction of two auxin hydrolases, which release active auxin from conjugate forms, has been found within 24 h of inoculation in *M. truncatula* (Campanella et al., 2008).

Retention of auxin in dividing cells might be mediated by flavonoids in the nodule precursor cells and early primordia. Certain flavonoids and simple phenolics can inhibit the action of peroxidases and auxin oxidases that break down active forms of auxin (Furuya et al., 1962; Grambow and Langenbeck-Schwich, 1983), and it has been shown *in vitro* that those flavonoids accumulating in the inner cortical cells of white clover inhibit auxin breakdown by peroxidase (Mathesius, 2001).

However, modeling suggested auxin transport regulation to be the more likely driver of creating auxin maxima in the cortex (Deinum et al., 2012; see Chapter 66). Localization of members of the auxin import protein family MtLAX showed that this transporter is strongly expressed in young nodule primordia in *M. truncatula* (de Billy et al., 2001). Similarly, the expression of the auxin export proteins PIN1 and PIN2 is localized in early nodule primordia in *M. truncatula*, and their silencing by RNAi leads to a reduction in nodule numbers (Huo et al., 2006). These studies strongly suggest a role of active auxin transport into the initiating nodule.

As the nodule primordium differentiates, auxin responses become restricted to peripheral cell layers of the primordium (see Fig. 47.1d). In mature nodules, auxin

responses are localized in vascular tissues, and the apical meristem and these expression patterns are similar to those in differentiating lateral roots (Mathesius et al., 1998b; Pacios-Bras et al., 2003). The expression pattern of the auxin importer *MtAUX1* is similar to the high expression in peripheral tissues of a nodule and central tissues of lateral roots, indicating that expression overlaps with regions of vascular tissue or endodermal differentiation (de Billy et al., 2001). As in lateral root formation, the changes in auxin responses suggest that auxin concentrations or responses must drop at the differentiation stage relative to the primordium initiation phase (Laskowski et al., 1995). The loss of auxin concentration in central parts of legume nodules could be regulated by peroxidases that destroy auxin accumulating inside the nodule (Fedorova et al., 2000; Mathesius, 2001).

The localization and role of auxin during legume nodulation differs from that in actinorhizal nodules. Examination of *AUX1* in *Casuarina glauca*, which forms nodules with *Frankia*, showed that its expression is localized to infected cells, first in the pre-nodule in cortical cells and later in the nodule (Peret et al., 2007). A possible role of the high auxin levels in infected cells has been suggested to be in controlling cell hypertrophy or cell wall remodeling during infection (Peret et al., 2007). However, *AUX1* expression is absent from nodule primordia, even though the same gene is strongly expressed in lateral root primordia in *Casuarina*. These studies suggest that actinorhizal nodules and lateral roots might require distinct auxin responses for their differentiation. Later studies have shown that a *PIN1*-like gene is specifically expressed in noninfected cells of the actinorhizal nodule (Perrine-Walker et al., 2010). Coupled with computational modeling data, the authors suggested that a PIN1-like and AUX1 protein in *C. glauca* act in concert to actively restrict auxin accumulation in *Frankia*-infected cells. Although isoflavones were found to play a role during *Frankia*-induced nodule formation in *C. glauca*, it remains unclear whether they are necessary for auxin transport regulation (Auguy et al., 2011). In the actinorhizal plant *Eleagnus umbellata*, high levels of an auxin-responsive protein have been found in the nodule-fixation zone, although it remains uncertain whether this expression pattern reflects actual auxin concentrations (Kim et al., 2007). The regulation of auxin transport and accumulation in actinorhizal plants remain unclear, and there might be dual roles of the symbiont and the host in synthesizing auxin as well as controlling auxin transport (Peret et al., 2007).

47.1.5 Auxin as a Long-Distance Regulator of Nodule Numbers

Nodule numbers are regulated by several mechanisms, including nitrogen availability and autoregulation. If sufficient nitrogen is available to the plant, nitrogen uptake from nitrate or ammonium is preferred over the costly establishment of a nitrogen-fixing symbiosis. Both nitrate and ammonium inhibit nodulation at stages of infection, nodule development, and nitrogen fixation (Streeter, 1988). The mechanisms of this inhibition and whether auxin is involved in this inhibition are still unclear. It has been shown that nitrate regulates lateral root initiation and elongation by both local and systemic mechanisms, and that this regulation involves auxin signaling, suggesting that similar mechanisms might be involved in nodulation (Walch-Liu et al., 2006).

The plant also limits nodule numbers on the whole root system by an internal, systemic regulatory mechanism termed autoregulation of nodulation (AON), which is dependent on the action of a leucine-rich repeat receptor-like kinase (NARK, nodulation autoregulation receptor kinase) acting in the shoot (Kinkema et al., 2006). After the root first perceives rhizobia, autoregulation inhibits further formation of nodules on later forming roots, probably to limit the amount of carbon redirected toward nodules. Split-root experiments have shown that an early event during nodule formation sends a signal to the shoot, where it, or a derivative signal, is perceived by NARK and causes a shoot-derived inhibitor (SDI) to move back to the root system to limit further nodulation (Kinkema et al., 2006). Because auxin is transported from the shoot, where most auxin is synthesized, to the root, the role of shoot-to-root transported auxin was investigated for its role in autoregulation. The *M. truncatula* autoregulation mutant *sunn1* (*supernumeric nodules1*) (Schnabel et al., 2005) transports significantly more auxin from the shoot to the root than the wild type (van Noorden et al., 2006), and auxin concentrations in the root zone susceptible to nodule initiation are increased in the *sunn1* mutant. In addition, the auxin-response gene *GH3* is expressed at much higher levels in inoculated *sunn1* than in wild-type roots (Penmetsa et al., 2003). Within 24 h of inoculating the root tip with compatible rhizobia, long-distance auxin transport from the shoot to the root is reduced in wild-type seedlings, at the same time as the start of autoregulation in *M. truncatula* (van Noorden et al., 2006). However, this inhibition of long-distance auxin transport does not occur in the *sunn1* mutant, suggesting that SUNN regulates long-distance auxin transport changes in response to inoculation. Treatment of the shoot–root junction of *sunn1* with NPA causes a reduction in nodule numbers to levels similar to the untreated wild type (van Noorden et al., 2006). Consistent with these findings, *DR5:GFP* expression in *L. japonicus* was reduced in the autoregulation mutant *har1* and also in the overexpression *LjCLE* of roots constitutively, encoding peptides that likely activate NARK signaling (Suzaki et al., 2012).

The long-distance regulation of auxin transport during AON in legumes forming indeterminate nodules is regulated independently of local auxin transport inhibition that occurs

at the root tip within hours of inoculation and is necessary for the initiation of the first nodules on the root (Fig. 47.2). The *sunn1* mutant shows local auxin transport inhibition after inoculation with rhizobia similar to the wild type, despite the difference in long-distance transport (van Noorden et al., 2006).

Nodule numbers are also regulated by ethylene, as demonstrated in the hypernodulation phenotype of the ethylene-insensitive *skl* (*sickle*) mutant (Penmetsa and Cook, 1997). The gene mutated in the *skl* mutant has been shown to encode an ortholog of the *Arabidopsis* ethylene signaling protein EIN2 (Penmetsa et al., 2008). Long-distance auxin transport was similar to that in wild type in uninoculated *skl* plants, whereas the downregulation of auxin transport observed in wild type 24 h after inoculation with rhizobia did not occur in *skl* (Prayitno et al., 2006). The relatively increased long-distance auxin transport in *skl* correlates with a higher number of nodules formed on the root, in a manner similar to the higher long-distance auxin transport in the supernodulating mutant *sunn1*.

The regulation of nodule numbers through AON is linked to the regulation of nodule numbers by nitrogen availability (Fig. 47.2). Studies in *M. truncatula* showed that if sufficient nitrate is available to the plant to inhibit nodule formation, this increases shoot-to-root auxin transport in uninoculated wild-type plants, but not in the *sunn1* mutant (Jin et al., 2012). After inoculation with rhizobia, auxin transport was reduced in plants growing under nitrogen-limiting conditions, but not under nitrogen-sufficient conditions. How the nitrogen status in the plant is "translated" into changes in auxin transport remains unknown. It is possible that auxin transport control by SUNN is a more general mechanism to control root architecture in response to nitrogen, because the *sunn1* mutant is also unable to control lateral root density in response to nitrate through the modulation of auxin transport (Jin et al., 2012).

47.2 CONCLUSIONS AND PERSPECTIVES

Many questions remain in our understanding of how rhizobia control host auxin transport. For example, it is unknown how flavonoids interact with the auxin transport machinery during nodule formation, where flavonoids act, and how they are transported from the site of induction to the site of auxin transport. It also needs to be tested whether flavonoids act in parallel or downstream of cytokinin signaling, and whether ethylene acts independently of cytokinin and flavonoids. Similarly, it is unknown how long-distance auxin transport is controlled through the action of SUNN. In addition, it is unclear how long-distance auxin transport acts in relation to the local auxin transport control. Examination of auxin transport mutants will be imperative for the further dissection of auxin transport control during nodulation. The study of auxin transport in *Arabidopsis* exploded with the characterization of auxin transport mutants; however, most of these are currently not available in legumes. In addition, it would be very interesting to study and compare auxin transport control in different species of legumes, actinorhizal plants, and in *Parasponia*, which all form different types of nodules.

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Chapter 48

Functional Analysis of Nitrogen-Fixing Root Nodule Symbioses Induced by *Frankia*: Transport and Metabolic Interactions

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48.1 INTRODUCTION

A diverse group of dicotyledonous plants belonging to 24 genera from eight different families can enter a symbiosis with *Frankia* spp., high G + C, Gram-positive soil Actinobacteria. *Frankia* strains induce the formation of root nodules, functionally complex organs specialized for biological nitrogen fixation (BNF), in symbiosis with the roots of their host plants. In these nodules, the microsymbionts, supplied with carbon by the host plant, fix dinitrogen and export the products of dinitrogen fixation to the host. Nitrogen supplied from symbiotic BNF can provide up to 100% of the host plant nitrogen requirement. Mature actinorhizal nodules are perennial organs consisting of multiple lobes, each of which represents a modified lateral root, with central vascular cylinder and cortex, and superficial periderm. The root nodule lobes retain an apical meristem, but lack a root cap. The microsymbionts are hosted inside expanded cells of the cortical tissue within the nodule lobes (see Chapters 24, 35, 42, 43, 55).

Actinorhizal families belong to three different orders (Fagales: Betulaceae, Casuarinaceae, and Myricaceae; Rosales: Elaeagnaceae, Rhamnaceae, and Rosaceae; Cucurbitales: Coriariaceae and Datiscaceae; see Fig. 48.1). Together with a fourth order, Fabales, which includes the legumes, these plant groups form the so-called nitrogen-fixing clade, a monophyletic subgroup within the Fabidae (Doyle, 2011). With one exception, *Dryas* (Rosaceae), all plants of an actinorhizal genus are symbiotic, but not all genera of an actinorhizal family are symbiotic. For example, within the Betulaceae, *Alnus* spp. are symbiotic but *Betula* spp. are not. There are parallels to this pattern of closely related nodulating and nonnodulating host genera in some basal groupings of the Fabaceae, for example, Cassieae (Caesalpinioideae) and certain members of the Mimosoideae (Sprent, 2009).

The phylogenetic evidence of relatedness (Soltis et al., 1995), together with the discovery that the legumes and actinorhizal plants share common signaling response pathways to inoculation with the microsymbiont (Markmann et al., 2008; Gherbi et al., 2008; see Chapters 42, 43, 55), indicate that the progenitor of this clade gained a unique property or set of properties, which allowed effective root nodule symbiosis (both rhizobial and actinorhizal) to evolve. It has been suggested that the invention of nodulation within the clade likely evolved multiple times (Swensen, 1996; Doyle,

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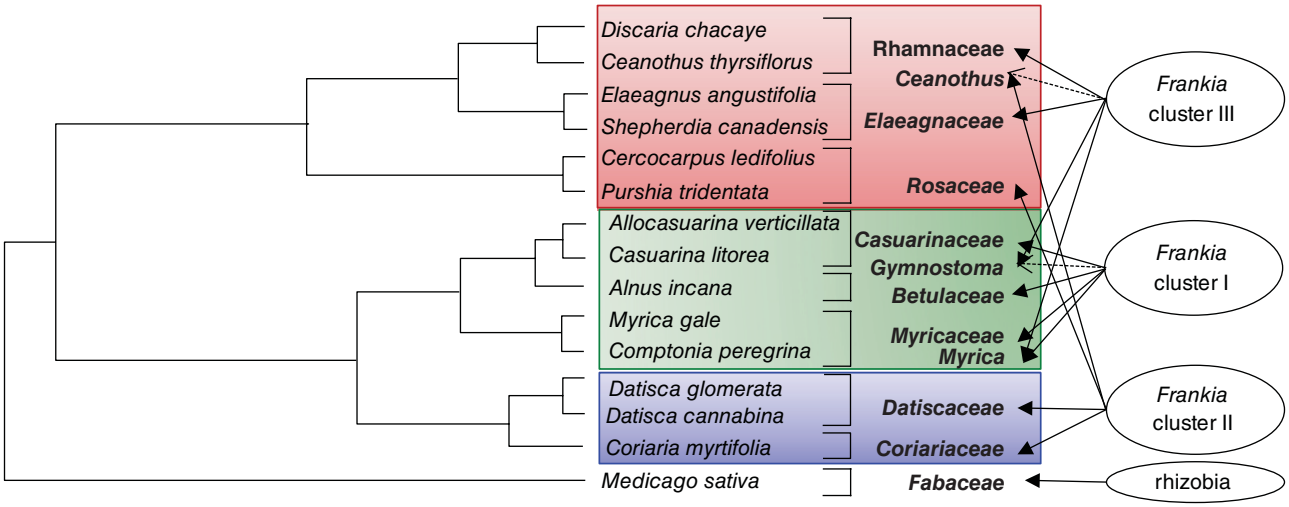

Figure 48.1 Phylogeny of the nitrogen-fixing clade and host specificity in actinorhizal symbioses are depicted in a simplified scheme adapted from Benson and Clawson [2000]. The actinorhizal plant families belong to three orders, Rosales (red box), Fagales (green box), and Cucurbitales (blue box). The numbering of *Frankia* clusters is adapted from Normand et al. [1996]. Thick arrows connecting a *Frankia* cluster with a group of plants indicate that members of this cluster are commonly found in nodules of these plants, while dashed arrows indicate that members of this cluster have been isolated from or detected in an effective or ineffective nodule of a member of this plant group at least once. Not all strains isolated from, or identified in, an actinorhizal nodule can reinfect the corresponding plant species (Ramirez-Saad et al., 1998).

2011). Furthermore, it has become apparent that elements of the evolutionarily much older and more widely distributed arbuscular mycorrhizal (AM) symbiosis between plants and Glomalean fungi were recruited and adapted as a basis for the initial steps in the host signaling pathway leading to root nodule symbiosis (Tromas et al., 2012; Markmann and Parniske, 2009; Oldroyd et al., 2009), although nodulation versus mycorrhizal symbiotic responses diverge after the early signaling steps (see Chapter 110).

Frankia grows vegetatively as septate branching hyphae with a diameter of 0.5–2 µm. Multilocular sporangia can form, containing thick-walled resting-type spores. *Frankia* fixes nitrogen in response to external nitrogen limitation not only in symbiosis but also in the free-living state, even under aerobic conditions, unlike most rhizobia. The reduction of dinitrogen to ammonium is catalyzed by nitrogenase, a highly O_2-sensitive enzyme (see Chapter 2), yet nitrogen fixation requires high amounts of ATP provided by aerobic respiration. This is the so-called "O_2 dilemma of nitrogen fixation." Under aerobic conditions, in response to nitrogen limitation, *Frankia* spp. strains solve this problem by forming vesicles: specialized thick-walled, spherical structures that differentiate on short hyphal stalks, usually side branches. Oxygen tension is regulated within the vesicles by high respiration rates (O_2 consumption). In addition, a multilayered envelope is deposited around the vesicle walls that comprises hopanoids, a class of bacterial sterol lipids (Berry et al., 1993). Such external hopanoid layers are not found in the hyphae. It is assumed that the vesicle envelope acts as a gas-diffusion barrier, supported by the fact that the number of layers comprising an envelope positively correlates with the external O_2 tension (Parsons et al., 1987), and by the packing behavior of artificial membranes containing different hopanoid concentrations (Stroeve et al., 1998). Under conditions of low-oxygen partial pressure, nitrogenase is synthesized in hyphae, and vesicles do not differentiate (Murry et al., 1985). Little is currently understood about the gene regulatory circuitry governing vesicle differentiation, or the biosynthesis of specialized hopanoid mixtures that accompanies vesicle formation. Although partial pressure of oxygen is a key environmental signal, no homologs of NifL–NifA are found in the sequenced *Frankia* genomes.

In symbiosis, within the nodule tissue, the shape, septation, and subcellular localization of *Frankia* vesicles, and whether vesicles are formed at all, depends on the host plant cell environment. In nodules of *Alnus* spp., ovoid septate vesicles are located in the outer parts of the host cytoplasm; in nodules of *Casuarina* spp., no vesicles are formed at all; whereas in nodules of Cucurbitales, long lanceolate vesicles are radially arranged around a central vacuole (Baker and Mullin, 1992; Silvester et al., 1999). Hence, as legumes, actinorhizal plants can direct the differentiation of their intracellular microsymbionts, either through modified environmental conditions (pH, pO_2, carbon, or nitrogen concentration) or possibly by molecular and/or cytoskeletal regulation.

48.1.1 *Frankia* Systematics

Based on phylogenetic analysis, the symbiotic *Frankia* strains can be grouped into three distinct clades or clusters (Normand et al., 1996). The members of one cluster (cluster

Table 48.1 Summary of *Frankia* genomes and selected major transporter groups

Genome	Genome Size (MB)	Total Transporters	Transporters per MB	Transporters % of Genes	ABC Type	Resistance-nodulation-cell division
ACN14a	7.5	559	74.5	8.2	278	11
CcI3	5.43	324	59.7	7	127	9
EAN1pec	8.98	628	69.9	8.7	335	22
EuI1c	8.82	759	86.1	10.4	411	16
Dg1	5.34	354	66.3	7.7	177	8

II) so far have never been cultured (Persson et al., 2011), but numerous isolate strains exist in clusters I and III. These three clusters also correspond to different host specificity groups, as shown in Figure 48.1. For example, members of cluster II are found in actinorhizal nodules of host plants in the Cucurbitales and Rosaceae, and in *Ceanothus* spp. (Rhamnaceae). Members of cluster III nodulate a broad range of host genera and families in Rosales and Fagales, whereas members of cluster I nodulate only within the Fagales. Patterns of host specificity also exist within a given *Frankia* cluster, in that not all members of a cluster can nodulate all plants associated with that cluster. For instance, several cluster I frankiae can only infect Casuarinaceae within the Fagales.

Genome size among the sequenced *Frankia* strains ranges widely, from about 5.3 Mb to over 9 Mb (Table 48.1). The smallest genome reported to date is that of the uncultured symbiont of *Datisca glomerata*, strain Dg1, in cluster II. The members of this cluster so far have never been cultured, so the genome was sequenced from DNA purified from *Frankia* cells derived from nodules (Persson et al., 2011). If cluster II frankiae are obligate symbionts, as they appear to be thus far, the small genome size likely indicates reduction due to loss of function. Otherwise within the frankiae, genome size has been correlated with the saprotrophic potential of *Frankia* strains and with host plant geographical distribution (Normand et al., 2007; see Chapter 24). The cluster III frankiae have the largest genomes, a wide host range, and are ubiquitously found in soils on all continents. In cluster I, the difference in genome size, for example, between ACN14a (host *Alnus* spp.) and CcI3 (host *Casuarina* spp.) may be attributable to the limited native distribution of *Casuarina*, primarily in Australia (Normand et al., 2007).

The availability of an increasing number of sequenced *Frankia* genomes makes it possible to begin comparative analyses pointing to functional properties, within and among the *Frankia* genomic clusters, and more broadly, between *Frankia* and rhizobia in the nitrogen-fixing symbioses.

48.1.2 Nutrient Exchange Between *Frankia* and Its Host Plant: *Frankia* Membrane Transporters

In symbiosis, intracellular bacteria depend on the host plants for many nutrients to support nitrogen fixation and survival within the plant cell environment, as summarized for legume symbioses (Oldroyd et al., 2011). Generally, the plant provides metal ions, sulfate, and carbon sources; the specific transport of host-derived branched-chain amino acids has also been demonstrated as a requirement for *R. leguminosarum* in symbiosis (Prell et al., 2009).

Characterization of transporter genes can provide one line of evidence of interactions between the bacteria and its environment, whether in symbiosis or in the soil (e.g., Young et al., 2006). In this chapter, we compare published genomes of five representative *Frankia* strains from the three clusters to characterize transporters. One set of *Frankia* transcriptomes, for the sequenced genome of ACN14a, has been published to date (Alloisio et al., 2010). This data set, based on microarray comparisons, allows further functional characterization of its transporters in symbiotic and free-living states.

ATP-binding cassette (ABC) transporter proteins are the most abundant class of transporters in the frankiae (Table 48.1). Substrates for identified transporters vary between strains (Table 48.2; transporter genes with substrates identified by annotation in databases BioCyc, KEGG, and IMG) such that many transporters lack a homolog in all sequenced genomes. Transporter types do not necessarily assort directly with clades, as benzoate transporters can be found in Clade II Dg1 (FsymDg_3355) and Clade III EuI1c (FraEuI1c_3834); glycine betaine/choline transporters are found in Clade I ACN14a (FRAAL6726) and EuI1c (FraEuI1c_0106), but not CcI3 and EAN1pec (Clades I and III, respectively). The ineffective strain EuI1c (parent strain EuI1 (Baker et al., 1980) GenBank accession no. ASM16613v1) that cannot form vesicles or fix nitrogen *in planta* has substantially more total transporters, and transporters as a percentage of the genome size, compared with the other four genomes (Table 48.1); this pattern holds across most categories of transporter genes. Many of the

Table 48.2 Annotated transporter classes in five *Frankia* genomes

	Frankia Strain					
	ACN14a	CCI3	Dg1	EAN1pec	EuI1c	Expressed in *A. glutinosa* Symbiosis
	Genome Locus Tag					
Transporter Substrate	FRAAL	Francci3_	FsymDg_	Franean1_	FraEuI1c_	
Saccharides						
Arabinose	—	—	—	—	3263	—
Lactose/Cellobiose	—	—	—	7072	—	—
Ribose	0205	—	1586	7025	5696	No
Rhamnose	—	—	—	—	3905	—
Trehalose	3763	—	—	4615	4049	Yes
Xylose	—	—	—	5185	7042	—
Amino Acids						
LAO Transporter	4949	3004	3821	1945	4944	Yes
Leucine/Isoleucine/Valine	See Figure 48.2					
Proline + Na+	4816	2471	—	0157	—	Yes
Glutamate/Glutamine	5715	3521	4250	1223	4296	Yes
Ammonium	0905	0422	0091	6204	2072	Yes
	1969				6378	
Other						
Actinorhodin	—	1120	—	—	—	—
Benzoate	—	3355	—	—	3834	—
Cytosine + purine	0376	—	—	—	6597	Yes
Dicarboxylate	1390	—	—	4413	—	Yes
Glycine betaine/choline	6726	—	—	—	0106	No
Indole acetic acid	1984	1249	—	2190	2188	Yes
Polyamine	6834	—	—	0023	0032	Yes
Urea	—	—	—	—	4792	—
Xanthine/uracil	6301	—	—	0733	3513	No

Four-digit numbers represent gene locus tags for each genome. Expression patterns for ACN14a transporter genes in symbiosis with *Alnus glutinosa* (column 8) are derived from GSE18190 (Alloisio et al. 2010) in the NCBI GEO database.

EuI1c transporter genes not found in other *Frankia* genomes appear to be derived from horizontal gene transfer (see Figs. 48.2 and 48.3).

48.1.3 Nutrient Exchange Between Host and Microsymbiont: Primary Nitrogen Metabolism

For legume–rhizobia symbioses, it has been shown that the first enzyme in primary nitrogen assimilation, glutamine synthetase, is repressed in the symbiotic bacteroids. Consequently, the bacteroids do not assimilate the fixed nitrogen themselves, but deliver it to the host plant as ammonium (summarized in Lodwig et al., 2003). In the plant cytosol, this ammonium is assimilated in the host glutamine synthetase–glutamate synthase (GS-GOGAT) cycle. This pattern of primary nitrogen assimilation is confirmed by the observed presence of high levels of plant cytosolic GS in the infected cells of legume nodules, and actinorhizal nodules from hosts in two different orders [*A. glutinosa* in Fagales: Hirel et al., 1982; Guan et al., 1996; and *Discaria trinervis* in Rosales: Valverde and Wall, 2003]. Thus, the dinitrogen fixed by *Frankia* is made available to the plant in the form of ammonium in these symbioses.

Both frankiae and rhizobia have two genes coding for glutamine synthetase (*glnA*, coding for GSI; and *gsII*). The *glnA* gene was strongly repressed in the *Alnus* sp.-infective strain ACN14a in symbiosis; *gsII* was expressed in nodules, but at a much lower level compared to free-living nitrogen-fixing cells (Alloisio et al., 2010). Genes coding for GOGAT were expressed in these nodules at low levels as well. An ammonium transporter was more highly expressed in *A. glutinosa* nodules than in ACN14a cells in culture. These findings indicate an active repression of microsymbiont GS in *A. glutinosa* nodules and ammonium efflux from the microsymbiont, with some low GS-GOGAT activity retained in frankiae *in planta*.

In *Datisca glomerata*, a representative of the Cucurbitales, infected by the cluster II strain *Candidatus* Frankia datiscae Dg1 (Dg1), nodule nitrogen assimilation is strikingly different from all other types of nodules examined thus

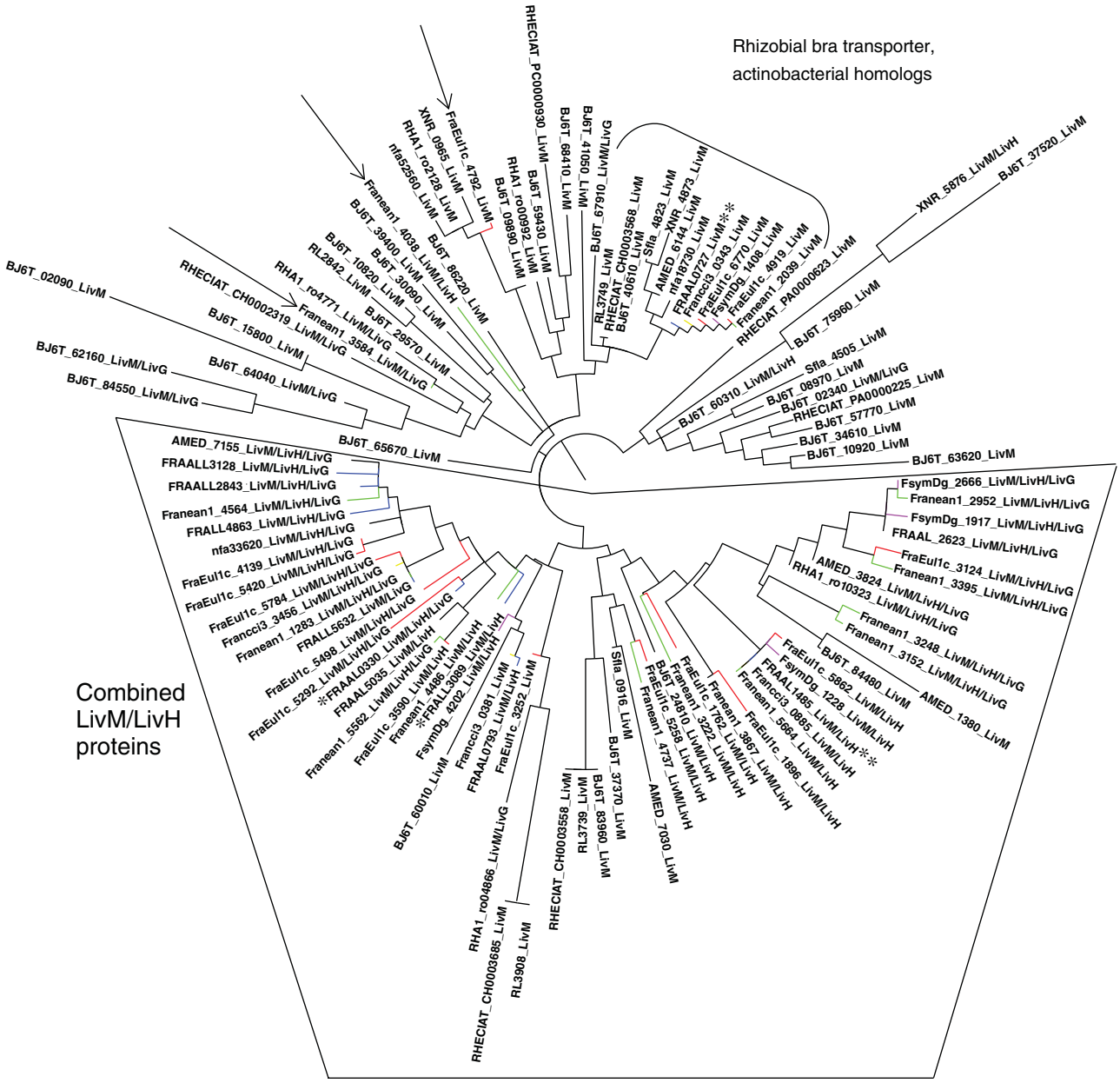

Figure 48.2 Maximum-likelihood protein tree of LivM-domains found in all five *Frankia* genomes and representative related species with full genome sequences. The clade that contains homologs likely related to the Bra transporter is labeled; as is the *Frankia*-dominated clade consisting of combined LIV-domain proteins. Arrows denote proteins that have likely been horizontally acquired in *Frankia*. Asterisks (*) denote FRAAL proteins whose genes are expressed at high levels in *A. glutinosa* symbiosis. Single star (*): Top 50%. Double star (**): Top 25% (GSE18190 in GEO). The tree was constructed using only the LIVM-domain from each protein, but all LIV-domains found in each protein are notated. Organism codes: FRAAL (ACN14a, colored blue), Francci3 (CCI3, colored yellow), Franean1 (EAN1pec, colored green), FraEuI1c (EuI1c, colored red), FsymDg (Dg1, colored pink), AMED (*Amycolatopsis mediterranei*), BJ6T (*Bradyrhizobium japonicum*), nfa (*Nocardia farcinica*), RHA (*Rhodococcus jostii*), RHECIAT (*Rhizobium etli*), RL (*Rhizobium leguminosarum*), Sfla (*Streptomyces flavogriseus*), and XNR (*Streptomyces albus*). Alignment generated in MUSCLE and tree calculated in MEGA v5.2.2.

far. Host plant GS accumulates not in the *Frankia*-infected cortical cells, but in the uninfected cortical cell layers surrounding the uniform patch of infected cells (Berry et al., 2004). A pattern of low relative gene expression was noted for *glnA* and *gsII* in nodules of *D. glomerata* using semiquantitative PCR (Berry et al., 2011), similar to the results for ACN14a, discussed earlier, by comparison with nodule *nifH* expression levels in each experiment. The gene coding for the small subunit of GOGAT was relatively strongly expressed in Dg1. In addition, genes

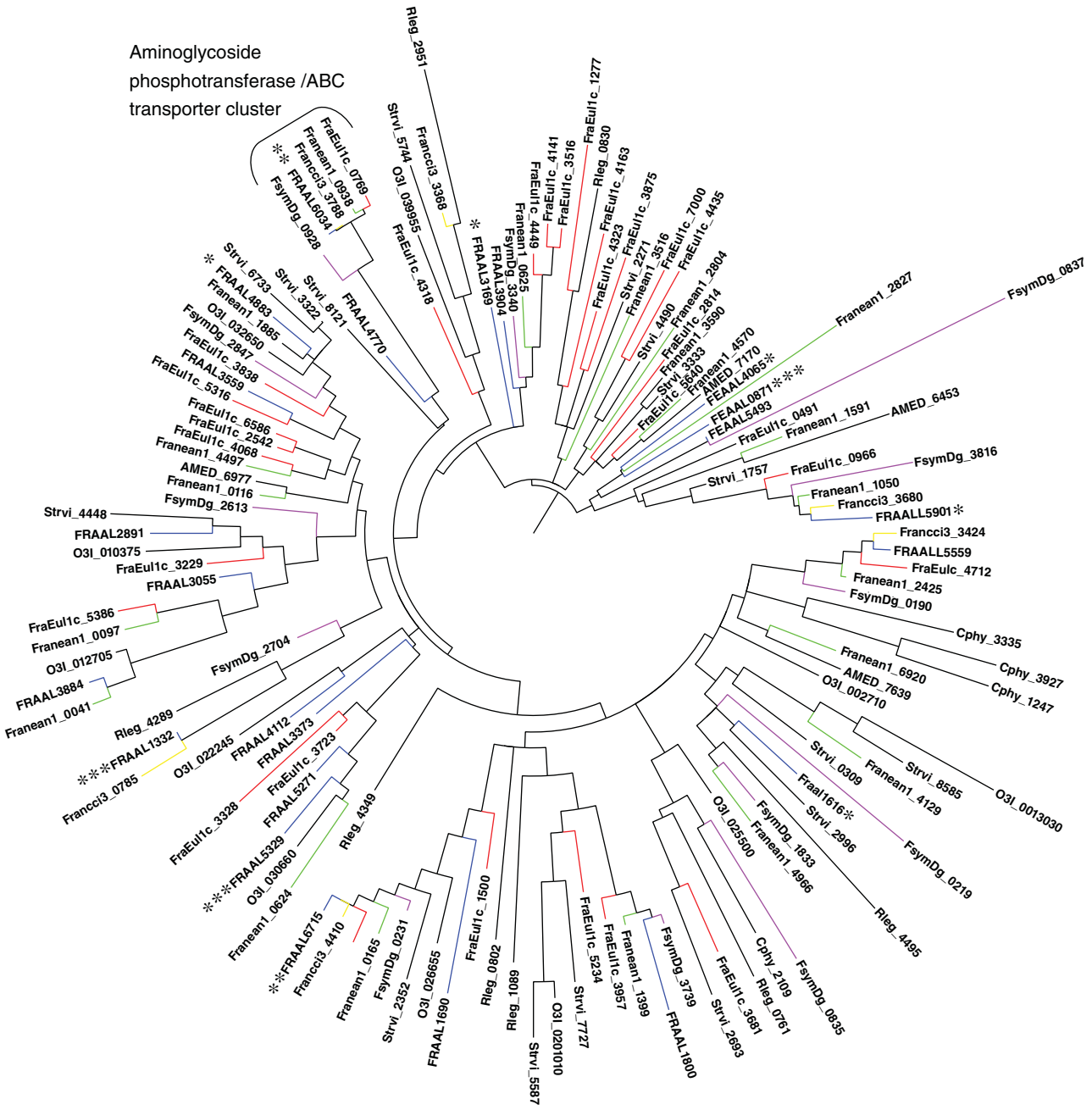

Figure 48.3 Maximum-likelihood protein tree of all putative aminoglycoside phosphotransferases found in all five *Frankia* genomes and representative related species with full genome sequences. The clade of proteins annotated as additionally containing ABC transport ATPase domain is labeled. Asterisks (*) denote FRAAL proteins whose genes are expressed at high levels in *A. glutinosa* symbiosis. Single star (*): Top 50%. Double star (**): Top 25%. Triple star (***): Top 10% (GSE18190 in GEO). Organisms codes: FRAAL (ACN14a, colored blue), Francci3 (CCI3, colored yellow), Franean1 (EAN1pec, colored green), FraEuI1c (EuI1c, colored red), FsymDg (Dg1, colored pink), AMED (*Amycolatopsis mediterranei*), Cphy (*Clostridium phytofermentans*), O3I (*Nocardia brasiliensis*), Rleg (*Rhizobium leguminosarum*), Strvi (*Streptomyces violaceusniger*). Alignment was generated in MUSCLE and the tree was calculated in MEGA v5.2.2.

for key enzymes in arginine biosynthesis were much more highly expressed in Dg1 than in ACN14a in nodules, suggesting that an active amino acid metabolism is operating in Dg1 in symbiosis.

Strikingly, amino acid profiles of the *D. glomerata* nodules revealed a high concentration of arginine (Berry et al., 2004), previously not reported as an abundant nitrogen-containing compound in any root nodule sym-

bioses, except nodules of *Coriaria* sp. (Wheeler and Bond, 1970), the other nodulating genus of the Cucurbitales. Arginine is an efficient temporary storage form of nitrogen, containing four nitrogens per molecule.

These lines of evidence suggest that in the Cucurbitales symbioses, ammonium is assimilated in *Frankia* into amino acids, including arginine, and then transported to the host tissue. The identity of the amine-containing compound or compounds exported from *Frankia* to the host remains to be discovered.

The evidence of high host GS activity in the uninfected cortical tissue likely indicates reassimilation of amino acids derived from *Frankia*. High GS activity implies the presence of free ammonium in the uninfected tissue. It is possible that this reassimilatory step is linked to an arginine–ornithine–urea cycle, a key step in storage-N mobilization in many plant taxa (Goldraij and Polacco, 2000) that produces free ammonium as a by-product; or conceivably free ammonium could result from deamination of glutamate by glutamate dehydrogenase, for TCA carbon skeleton regeneration (Miyashita and Good, 2008).

This specialization of nodule nitrogen metabolism does not seem to be linked to the nitrogen transport form in the xylem: *D. glomerata* transports nitrogen in the xylem in the form of amides, similar to other actinorhizal plants except *Alnus* spp. and certain *Casuarina* spp., who export amides and citrulline to the host xylem (Valverde and Huss-Danell, 2008).

48.1.4 Nutrient Exchange Between Host and Microsymbiont: Transport of Branched-Chain Amino Acids (LIV), Arginine, and Polar Amino Acids

In legume–rhizobia symbioses, the microsymbionts depend on the plant for supply of branched-chain amino acids via the Aap and Bra systems of bacterial transporters (Prell et al., 2009). This auxotrophy is not present in the saprotrophic state; rather, rhizobial genes encoding enzymes involved in the synthesis of branched-chain amino acids are downregulated in symbiosis. Because of the importance of this exchange in nitrogen-fixing symbiosis, we analyzed the LIV genes (leucine–isoleucine–valine), coding for branched-chain amino acid transport, in the *Frankia* genomes. All five of the *Frankia* genomes analyzed, including the symbiotically ineffective strain EuI1c, contain multiple LIV gene systems (Table 48.3). In symbiosis with *A. glutinosa*, one of the LivK (substrate-binding) genes of ACN14a (FRAAL0725) is substantially upregulated, compared with free-living, nitrogen-fixing cells (Alloisio et al., 2010) (GEO DataSet GSE18190 in NCBI); and a LivK protein was detected in nodules of *Alnus glutinosa* (ACN14a) and *Casuarina glauca* (CcI3) by Mastronunzio et al. [2009]. In ACN14a, this gene copy of *livK* is located adjacent to the other genes for the Bra branched-chain amino acid transporter (GI:4231701), which are also highly expressed in symbiosis, suggesting that a Bra operon is important in symbiotic nitrogen exchange in *A. glutinosa* nodules. The Bra transport system is not strictly specific for transport of branched-chain amino acids, but could also be involved in transport of polar amino acids and γ-aminobutyric acid (GABA) (Hosie et al., 2002). Thus, the identity of the LivK substrate(s) of the *Frankia* Bra transporter in actinorhizal symbiotic interactions remains to be demonstrated; nevertheless, in *Rhizobium*, it is the transport of branched-chain amino acids through Bra that seems to be required for symbiosis (Prell et al., 2009).

We further categorized the *Frankia* LIV transporter genes based on the presence of LivM-like and/or LivH-like domains, the two transmembrane domains. In addition, these ABC transporters have LivG and LivF ATPases, and the LivK substrate-binding domain was discussed earlier (Table 48.3). ABC transporters are known to contain fused genes that combine a transmembrane domain with an ATPase domain (Davidson et al., 2008). However, in *Frankia*, most of the LIV gene-encoded proteins are composed of a variety of combinations of LivM, LivH, LivG, and/or LivF domains combined into single proteins by gene fusion, even the two transmembrane domains, an unusual arrangement for ABC transporter gene fusions (Fig. 48.2). LivM and LivH were used to create protein trees either from the single-domain proteins or from the full multidomain protein sequences by taking the individual domains (Fig. 48.2). The multidomain ("combined") LIV genes are the most abundant in the *Frankia* genomes and appear to have originated primarily in Actinobacteria; moreover, ACN14a, EAN1pec, and EuI1c each have more genes with LivM/LivH combined domains than any other sequenced organism in the NCBI database. No LivM/LivH combined genes are annotated among rhizobial transporters. Interestingly, on the other hand, the clade of LivM-only proteins for all five *Frankia* genomes clusters closely with the rhizobial Bra transporter (shown in Fig. 48.2), suggesting horizontal gene transfer events between Actinobacteria and Proteobacteria, and further suggesting the possible identity of this group as the *Frankia* Bra transporters. The LivM-only protein (i.e., the potential Bra transporter permease) in ACN14a (FRAAL0727) is expressed equivalently in both symbiotic and nonsymbiotic conditions (top 25% of genes expressed), although LivK in the same operon is upregulated in symbiosis (top 5% of genes expressed) (Alloisio et al., 2010). Additionally, FRAAL0330 and FRAAL1485 are LIV-transporters (LivH/LivM/LivG and LivH/LivM fusions, respectively) that are expressed at high levels in symbiosis, in the top 40% and 20% of genes (GSE18190 in GEO), respectively.

Exceptions to these two major groups include Francci3_0381, a LivM-only protein that groups in the multidomain

Table 48.3 LIV-domain-containing genes in *Frankia* Genomes

Genome	Genes Containing					Total LIV Genes
	LIVM	LIVH	LIVG	LIVF	LIVK	
ACN14a	11	13	14	12	3	34
CcI3	4	2	4	4	3	13
EAN1pec	15	16	31	31	3	44
EuI1c	15	18	18	16	3	49
Dg1	5	5	5	4	4	17

clade; and a few proteins from EAN1pec and EuI1c that appear to be horizontally transferred from the Gammaproteobacteria (Fig. 48.2).

Other amino acid transporters with database annotations include those for arginine, proline, and polar amino acids. The identified arginine transporters correspond to the ATPase of LAO transporters, responsible for lysine, arginine, and ornithine transport (FRAAL4949, Francci3_3004, Franean1_1945, FraEuI1c_4944, and FsymDg_3821). This transporter is twofold upregulated in ACN14a in symbiosis (in the top 25% of genes in microarray data), compared with free-living conditions (GSE18190 in GEO), implying a role for one of these amino acids in the nutrient exchange between plant and bacterium in *A. glutinosa* nodules. The permease and substrate-binding proteins have not been specifically identified in any of the genomes. The ACN14a proline transporter is expressed in symbiosis in the top 45% of genes (GSE18190 in GEO).

Two major clusters of polar amino acid ABC transporters were found in all five sequenced *Frankia* genomes under discussion here. Based on UniProt characterization, these transporters are responsible for glutamate and glutamine transport (as well as aspartate and asparagine transport). These proteins are apparently primarily vertically derived, forming a tight *Frankia* clade (gene tree not shown). In ACN14a, one of these genes is expressed in symbiosis (FRAAL5715, expressed in the top 40% of genes), although it is downregulated compared with free-living, nitrogen-fixing conditions; the other genes are expressed only at very low levels (GSE18190, in GEO). Other polar amino acid transporters lack more specific annotation (FsymDg_4250, FraEuI1c_3110, FRAAL5987, and FraEuI1c_0815); these appear to have been horizontally transferred from other Actinobacteria based on their scattered distribution in the UniProt-based gene tree (not shown).

48.1.5 Nutrient Exchange Between Host and Microsymbiont: Carbon Metabolism

For legume nodules, it is widely accepted that dicarboxylates are the carbon sources supplied by the plant to the microsymbionts – mostly malate and also fumarate and succinate – although the plant exporter has not been identified yet (summarized by White et al., 2007). Physiological studies on *Frankia* vesicle clusters isolated from *Alnus glutinosa* nodules indicated that malate was likely to be the carbon source for intracellular *Frankia* as well (reviewed by Huss-Danell, 1997). A nodule-specific dicarboxylate exporter from *A. glutinosa* has been identified and located at the symbiotic interface (Jeong et al., 2004). The ACN14a genome contains a dicarboxylate transporter gene, *dctA*, which is expressed in symbiosis, but downregulated relative to free-living conditions (Alloisio et al., 2010; GSE18190 in GEO). A homolog of *dctA* occurs in EAN1pec, but not in CcI3 or Dg1, suggesting that other carbon transport mechanisms may be operating in at least some actinorhizal symbioses. DctA is not the only bacterial dicarboxylate transporter known; yet, so far no homologs of the other known dicarboxylate transporters (Youn et al., 2008; Valentini et al., 2011; Thein et al., 2012) have been identified in the published *Frankia* genomes. At any rate, since *dctA* was not found in CcI3 or Dg1, either the carbon sources supplied in actinorhizal symbioses are very diverse, or some frankiae use a dicarboxylate transporter that has not been characterized yet.

Which other carbon sources could be provided? In the saprotrophic state, clade I frankiae are able to grow well on short-chain fatty acids (acetate and propionate), variably on succinate, malate, or pyruvate, and poorly or not at all on different sugars, while *Elaeagnus*-infective strains grow on sugars as well as organic acids (Benson and Silvester, 1993). Since short-chain fatty acids do not seem to be part of plant exudates or cell–cell carbon exchanges, sugars might be worth an examination. In this context, it is interesting that *D. glomerata*, particularly in the root nodules, utilizes a rather peculiar carbon metabolism: the disaccharides rutinose and methylrutinose – α-L-rhamnopyranoside-(1→6)-D-glucose and α-L-rhamnopyranoside-(1→6)-1-*O*-β-D-methylglucose are used as intermediary carbon storage forms (Schubert et al., 2010). The same disaccharides were also found in *Datisca cannabina*, though not in *Coriaria* sp.; however, since rutinose levels seemed to vary with the growth conditions [Voitsekhovskaja and Pawlowski, unpublished observations], the lack of rutinose in *Coriaria* sp. might

have presented an artifact. So at least two actinorhizal members of the Cucurbitales contain high amounts of rhamnose-containing disaccharides in their roots and nodules.

Two types of disaccharide transporters are found in all five *Frankia* genomes. Genes belonging to COG0395 ("UgpE; ABC-type sugar transport system, permease component [carbohydrate transport and metabolism]") were identified and combined into a tree (data not shown). One cluster of *Frankia* transporters (from ACN14a, EAN1pec, and EuI1c) corresponded to *Mycobacterium* SugB, identified as a trehalose importer (Kalscheuer et al., 2010). The ACN14a homolog was FRAAL3763, which is strongly downregulated in the transcriptome in symbiosis (GSE18190 in GEO).

Additionally, in this COG, there is a clade of transporters in all five genomes with MalK maltose/trehalose/maltodextrin and/or glycerol-3-phosphate transporter annotations. Neither of the two *malK* homologs in ACN14a (FRAAL3201 and FRAAL4369) are highly expressed, either in nodule symbiosis or in free-living conditions; yet, this may be related to the fact that in *Alnus*-nodulating *Frankia*, the main carbon sources are not sugars (Jeong et al., 2004). The other four genomes have two proteins in this clade apiece (Francci3_2254 and Francci3_2838, Franean1_1897 and 3946, FraEuI1c_2623 and 4704, and FsymDg_2865 and 3447). This protein family might represent a candidate for a widespread saccharide uptake transporter in *Frankia*. Altogether, at the present stage, we cannot say whether (a) frankiae contain an as yet unknown class of dicarboxylate transporters or (b) carbon sources provided to frankiae *in planta* differ between host species.

Most rhizobia lack the homocitrate synthase gene [*nifV*; Hakoyama et al., 2009] and therefore have to import homocitrate, an essential part of the FeMo cofactor of nitrogenase, from the plant. So far, all of the sequenced genomes of symbiotic *Frankia* strains contain *nifV*, as would be expected since effective *Frankia* strains can fix nitrogen not only *in planta* but also in the saprotrophic state. Consistent with the function of NifV in nitrogen fixation, only the nonsymbiotic, nonnitrogen fixing strains CN3 (Ghodhbane-Gtari et al., 2013) and the symbiotically ineffective strain EuI1c lack a *nifV* homolog.

48.1.6 Nutrient Exchange Between Plant and Microsymbiont: Secondary Compound Transporters

48.1.6.1 Indole Acetic Acid (IAA). A gene coding for an auxin efflux transporter (IAA transporter) is found in four of the five genomes, absent only in Dg1. In the *A. glutinosa* symbiosis, this gene is upregulated compared to free-living conditions (GSE18190 in GEO). Genes for IAA biosynthesis are found in all four of the IAA transporter-containing genomes, but are absent in Dg1. This suggests that if IAA plays a role in symbiosis, as indicated by gene upregulation in *C. glauca* nodules (Perrine-Walker et al., 2011), there must be an alternative mechanism in the nodules of Cucurbitales. All five genomes encode genes for the enzymes of the biosynthetic pathway for phenylacetic acid (PAA), another type of auxin. PAA, which has been shown to be produced by *Alnus*- and *Elaeagnus*-infective strains (Hammad et al., 2003) and by CcI3 (Perrine-Walker et al., 2011) does not require a transporter since it is membrane-permeable (Simon and Petrášek, 2011).

48.1.6.2 Purine Transporters. A symporter of cytosine and a purine is present in two genomes, ACN14a and EuI1c, but no homolog is present in the other three genomes; it is twofold upregulated in the ACN14a nodule transcriptome, placing it in the top 25% of genes in symbiosis (Alloisio et al., 2010). A second purine transport-related gene, annotated as xanthine/uracil/vitamin C permease, but likely with broader specificity (Loh et al., 2006), occurs in ACN14a, EAN1pec, and EuI1c; it is weakly expressed in ACN14a in either free-living or symbiotic conditions.

48.1.6.3 Polyamine (Putrescine/Spermidine) Transporters. Polyamine production, especially putrescine production, was detected in several *Frankia* strains (Wheeler et al., 1994), and putrescine is commonly found in nodules and roots of some actinorhizal plants (reviewed in Valverde and Huss-Danell, 2008). In the legume *Lotus japonicus*, plant genes involved in polyamine biosynthesis via ornithine decarboxylase (LjODC), but not via arginine decarboxylase (LjADC), are upregulated in the early stages of nodule development (Flemetakis et al., 2004), implying a role in nodulation. All five *Frankia* genomes contain bacterial homologs of the plant protein LjODC, with relatively low e-values. All five genomes have genes coding for the conversion of agmatine to putrescine (the arginine pathway). Three of the five *Frankia* genomes contain annotated polyamine transporters (Dg1 and CcI3 do not). The polyamine transporter in ACN14a is upregulated twofold and highly expressed in symbiosis (top 15% of genes; GSE18190 in GEO), indicating its importance for ACN14a in symbiosis. Since nitrogen assimilation activity in ACN14a *in planta* seems to be very low, this would likely indicate uptake, not export. Yet, the absence of an identifiable polyamine transporter in CcI3, which nodulates the closely related *Casuarina* genus, makes it doubtful that polyamine uptake is essential for *Frankia* in all of the actinorhizal symbioses.

48.1.6.4 Resistance-Nodulation-Cell Division Efflux Pumps. Genes encoding efflux pumps of the resistance-nodulation-cell division family (RND), which

in rhizobia are involved in increasing symbiotic efficiency (Lindemann et al., 2010; Alvarez-Ortega et al., 2013), can be identified in all five genomes. Two out of three RND efflux exporters identified by Alloisio et al. (2010) were strongly upregulated in symbiosis, among the most highly expressed genes, making it likely that the encoded efflux pumps have a symbiotic function in the *A. glutinosa* nodule as well.

48.1.6.5 Aminoglycoside Phosphotransferases/Transporters. A very large family of presumptive aminoglycoside phosphotransferases exists throughout all sequenced *Frankia* genomes (Fig. 48.3), and homologs of the phosphotransferases are abundant in many actinobacterial genomes. Aminoglycosides *sensu stricto* are categorized as a class of antibiotics. Across the five *Frankia* genomes, the number of these proteins varies from 6 (CcI3) to 35 (EuI1c), a result of both gene duplications and horizontal transfer from other Actinobacteria, based on the phylogenetic data (Fig. 48.3). Though the majority of these proteins contain only the phosphotransferase domain, a few larger members (FRAAL6034, Francci3_3788, Franean1_0938, FraEuI1c_0769, and FsymDg_0928) have annotations corresponding to ABC transporter ATPases as well. These proteins form a tight and highly conserved cluster when placed on a tree with the other members of the family, as in Figure 48.3. Unfortunately, the remaining aminoglycoside phosphotransferase genes have been assigned a "transporter" annotation in several databases, based on homology among the phosphotransferase domains, even though most lack evidence of membrane association. In ACN14a, the majority of these genes (including the putative transporter) are downregulated or equally expressed in symbiosis, compared with free-living conditions, with the exception of FRAAL4883 and FRAAL6715, which are in the top 40% and 25% of genes expressed in the *A. glutinosa* symbiosis, respectively (GSE18190 in GEO). Despite the relative lack of upregulation in the ACN14a symbiosis compared with the free-living state, some of the other aminoglycoside phosphotransferases still remain very highly expressed: FRAAL0871, FRAAL1332, and FRAAL5329 are each in the top 10% of gene expression in symbiosis; and the putative transporter is in the top 25% (GSE18190 in GEO). Some homologs of these aminoglycoside phosphotransferases are annotated in genomes of some rhizobia (*R. leguminosarum* proteins are included in Fig. 48.3); however, their distribution varies between species and genera ranging from 1 to 11. Of interest, *Bradyrhizobia* have a homolog of FRAAL4883 (e-value around e-55), FRAAL0871 (e-09), and FRAAL5329 (e-12), but *Rhizobium* and *Sinorhizobium* do not. All three genera have homologs of the putative transporter (e-15 to e-45), but they align only over the transporter domain.

48.2 CONCLUSIONS

In order to understand the nutrient exchange in actinorhizal symbioses, detailed studies of plant and bacterial symbiotic transcriptomes are a logical next step. Although an increasing number of sequenced *Frankia* genomes are available, transporters are not yet fully annotated in existing bacterial genome databases. Biochemical evidence of bacterial transporter specificity is needed, especially for the Actinobacteria. As pointed out for rhizobia {Ramachandran et al., 2011; Udvardi and Poole, 2013], many *Frankia* transporters are likely to be of functional significance in soil and rhizosphere under saprotrophic conditions, to a greater degree than in the specialized exchanges during symbiosis within the nodule. This likelihood is reinforced by the finding of the highest number of transporters in EuI1c, which is symbiotically limited.

The *Frankia* transporters that we have characterized with potential significance in the symbiotic exchange include LAO transporters and LIV transporters. Several aminoglycoside phosphotransferases including the transporter-containing protein appear to play a significant role in nodule symbiotic interactions based on the ACN14a/*Alnus glutinosa* gene expression data. Several genes known to be involved in rhizobial symbioses have close homologs in *Frankia*, which are expressed in symbiosis, at least in *Alnus glutinosa* nodules. An avenue for future inquiry will be the directionality (or directionalities) of horizontal gene transfer to explore how the phylogenetically distant microsymbionts developed the capacity to form the root-nodule symbioses. Arginine metabolism appears to play an important role in symbiotic nitrogen assimilation in the Cucurbitales, but the precise patterns of exchange and functional compartmentalization await further research. The specific substrates for carbon exchange between host and microsymbiont in actinorhizal nodules remain for the most part unknown, and may represent a diversity of adaptations, in both the host and *Frankia* lineages.

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Chapter 49

NOOT-Dependent Control of Nodule Identity: Nodule Homeosis and Merirostem Perturbation

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ABBREVIATIONS

NSP	Nodulation Signaling Pathway
SYM	SYMbiotic pathway
CSP	Common Symbiotic Pathway
RNS	Root Nodule Symbiosis
SAM	Shoot Apical Meristem
RAM	Root Apical Meristem
OC	Organizing Center
QC	Quiescent Center
SCN	Stem Cell Niche
LR	Lateral Root
BOP	BLADE-ON-PETIOLE
COCH	COCHLEATA
NOOT	NODULE ROOT
NPR1	NON-EXPRESSOR OF PATHOGENESIS-RELATED PROTEIN 1
NBCL	NOOT BOP COCH LIKE

49.1 INTRODUCTION

Although a vast majority of the green lineage enters a mycorrhizal symbiosis with soil fungi from the *Glomeromycota* clade (Smith and Read, 2008), the ability to develop specialized symbiotic nitrogen-fixing organs is restricted to a small portion of Angiosperms called the nitrogen-fixing clade (Soltis et al., 1995). The large majority of these symbiotic organs, known as nodules, are root-derived organs characterized by peripheral vasculatures in legume plants or centrally located vasculature in actinorhizal plants and *Parasponia andersonii* (Fig. 49.1). For this reason, legume nodules are often considered as organs *sui generis* in contrast to the root-derived nature of actinorhizal and *P. andersonii* nodules (Hirsch and Larue, 1997).

The anatomy of the different types of symbiotic nodules greatly varies even inside legumes (Sprent, 2007), where two main nodule classes have been defined depending on the presence or absence of a persistent meristem (indeterminate *vs.* determinate nodules, respectively). However, despite this diversity, it appears that conserved signaling modules are triggered and/or necessary for nodule formation in and between the three nodulating groups of plants (Gherbi et al., 2008; Op den Camp et al., 2011a; Tromas et al., 2012). It is now well accepted that nodulation signaling pathway (NSP) evolved by the co-option of mycorrhization molecular machinery, thus defining a common symbiotic signaling pathway (CSP, CSSP or SYM) (Parniske, 2008; Kouchi et al., 2010; Gough and Cullimore, 2011; see Chapters 41–43, 55, 110). The NSP thus allows the infection of host cells by the bacterial partner and also triggers an orig-

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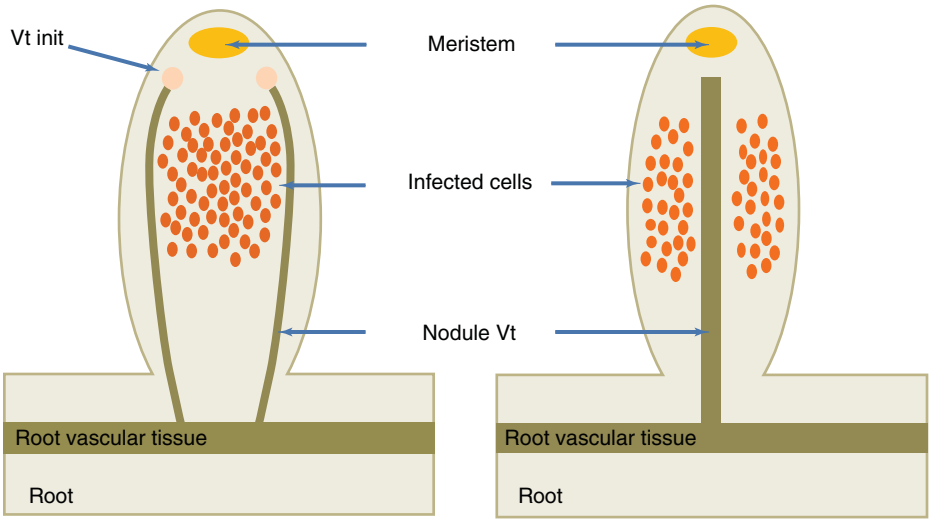

Figure 49.1 Schematic representation of indeterminate-type and actinorhizal nodules. (a) This represents an indeterminate legume nodule with peripheral vasculature (Nodule Vt) and centrally located symbiotic infected cells (red/orange dots). (b) This represents an actinorhizal type of nodule with a central vasculature (Nodule Vt) and peripheral symbiotic cells (red/orange dots). The position of the nodule meristem is indicated for the two nodule types. In the legume nodule, the position of the vascular initials (Vt Init) that can give rise to the roots in the mutant backgrounds is also indicated at the tip of the vascular tissue.

inal developmental program. Although genetically distinct, infection and organogenesis represent two tightly coordinated processes necessary for nodule formation (Oldroyd and Downie, 2008). Over the past decades, a great deal of knowledge has been acquired concerning processes such as infection, early organogenesis, autoregulation of nodulation, bacterial differentiation, and nitrogen fixation (Ferguson et al., 2010; Batut et al., 2011; Desbrosses and Stougaard, 2011; Oldroyd et al., 2011; Mortier et al., 2012). However, we do not know much about the origin of these organs acquired recently in the evolutionary history of land plants (Soltis et al., 1995) and how the nodule(s) developmental program(s) is (are) related to each other and to those of others organs (Hirsch and Larue, 1997).

Plant organs are generally built by the activity of meristems. The shoot apical meristem (SAM) and root apical meristem (RAM) define the main body of the plant (Stahl and Simon, 2010). These meristems fulfill two main functions: (i) self-maintenance and (ii) organogenesis. The organizing center (OC) or quiescent cells (QC) are groups of low mitotically active cells that are centrally located in the SAM and the RAM, respectively. Each is surrounded by a small group of stem cells, defining the stem cell niche (SCN), which combines division and self-maintenance activities. The activity of these SCN is highly regulated and is necessary to furnish all the cell types necessary for building the primary shoot or root. In the SAM, the stem cells are distally located in the meristematic dome, while in the RAM they are subapically located and protected by a root cap in the rootward orientation. The *Arabidopsis thaliana* RAM, which was used to define the model RAM structure, is of the closed type while legume plants have an open RAM (Groot et al., 2004; Heimsch and Seago, 2008). In plants with a closed RAM, each cell type of the root can be connected to one initial stem cell (Dolan et al., 1993). In contrast, in plants with open meristems, the origin of the different cell types of the root cannot be connected to one initial cell. Note that the basic open RAM organization is principally found in Fabaceae and Cucurbitaceae (Rost, 2011), two plant families that are able to generate root nodules. Another important difference in terms of lateral root (LR) formation exists between the model system *A. thaliana* and legume plants such as *L. japonicus* and *M. truncatula*. In *A. thaliana*, LRs exclusively originate from a small group of pericycle founder cells (Malamy and Benfey, 1997; Casimiro et al., 2003). However, this pattern of LR formation is not universal. In legume plants, LRs are also formed predominantly from the pericycle cells (Mallory et al., 1970), but this process also involves other root layers such as the cortex in *L. japonicus* and *M. truncatula* (Op den Camp et al., 2011b).

In contrast to the well-described RAM and SAM model systems in *A. thaliana* (Stahl and Simon, 2010), less is known about the functioning of legume RAM (open type) and even less about the molecular mechanisms governing the functioning, maintenance, and identity of the nodule meristem. If nodule and root organogenesis use common regulatory mechanisms (Hirsch and Larue, 1997), we do not know how the presence of the open-type RAM is of importance for nodule organogenesis. The persistence of an active meristem in the indeterminate nodule (Fig. 49.1) suggests the existence of a SCN(s) in this organ. We can also postulate that the balance between division and differentiation is tightly regulated during nodule organogenesis, as nodules stop developing and enter senescence weeks or months after their initiations depending on the species. However, we do not know how this (these) SCN(s) are distributed and function during nodule development.

Homeotic mutants represent powerful tools to study relationships between different organogenetic pathways. The study of homeotic traits has allowed, for example,

building the floral ABC model in Angiosperms (Coen and Meyerowitz, 1991; Causier et al., 2010) and the Hox-gradient model in *Drosophila melanogaster* (McGinnis et al., 1984; Scott and Weiner, 1984). It has long been suspected that a certain degree of homology exists between roots and nodules (Nutman, 1948; Nutman, 1949; Nutman, 1952; Libbenga and Harkes, 1973). However, the lack of homeotic mutant for the nodule made this question difficult to answer (Hirsch and Larue, 1997), until the first report of nodules converted into roots in the pea *cochleata* mutant (Voroshilova et al., 2003; Ferguson and Reid, 2005).

We have recently characterized the pea *cochleata* and the Medicago *noot* (*nodule root*) recessive mutants as being mutated in orthologous genes (Couzigou et al., 2012). The mutation of a single gene resulting in nodule to root homeosis indicated for the first time that the root is a component of the default identity of the legume nodules, and that this gene acts in repressing (part of) this root identity in the symbiotic organ. In addition to the formation of a root in the apical position of the nodule, the *noot* and *coch* mutants are characterized by enlarged nodule meristems and alteration of stipule and flower developments (Wellensiek, 1963; Gourlay et al., 2000; Yaxley et al., 2001; Couzigou et al., 2012). The legume *NOOT* and *COCH* genes are orthologs of the well-characterized *A. thaliana BLADE-ON-PETIOLE* (*BOP*) genes (Hepworth et al., 2005; Norberg et al., 2005), also necessary for the definition of the leaves and flowers. The identification of the *NOOT* and *COCH* genes as *BOP* orthologs defines a new clade (*NBCL* clade) in the BTB/POZ-ankyrin domain proteins sharing conserved functions in dicot aerial organ development (Couzigou et al., 2012). The monophyletic NBCL clade groups with a sister clade are made of NPR1-LIKE proteins (NPR1 to 4 in *Arabidopsis*) and is supported by a high level of synteny detected between the *NBCL* loci in several plants (Couzigou et al., 2012). This common *NBCL* origin is also supported by their conserved functions (regulation of stipule, flower, inflorescence, foliar senescence, and abscission) in distant Angiosperm species [Arabidopsis, Medicago, Lupin, Tobacco, pea – Hepworth et al., 2005, Norberg et al., 2005, McKim et al., 2008; Khan et al., 2012; Wu et al., 2012; Couzigou and Ratet, unpublished]. In the distant model moss *Physcomitrella patens*, *BOP* genes are also developmental regulators during juvenile-to-adult gametophyte transition (Saleh et al., 2011). In the light of these data, a hypothesis of functional orthology might be favored following a parsimonious hypothesis. Indeed, it is unlikely that all these common functions exerted by NBCLs in distant species are only due to independent convergence mechanisms.

In this chapter, we discuss the role of the *NOOT* and *COCH* genes during nodule organogenesis in Medicago and pea, respectively. Their principal symbiotic functions in indeterminate nodules discussed earlier are (i) the maintenance of a harmonious meristematic growth and (ii) the repression of ectopic root formation from nodule vascular initials (Fig. 49.2a–f). These two symbiotic roles of *NOOT* and *COCH* parallel the *BOP* functions proposed by Norberg et al. (2005) for shoot development in *A. thaliana* and are in agreement with the functional orthology hypothesis. In this study, the authors proposed that the BOP proteins are necessary (i) for the maintenance of a harmonious meristematic activity (e.g., inflorescence architecture) and also (ii) for the repression of ectopic organs formation (e.g., bract suppression). In the following, we also discuss the induction of similar modified nodules from various examples of the literature and a hypothetical cross talk between *NOOT* and *COCH* and plant defense reactions during nodulation.

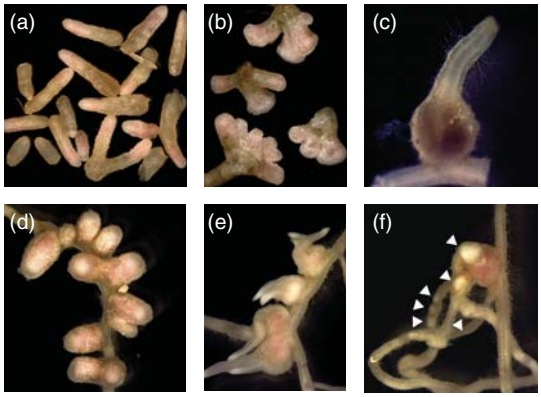

Figure 49.2 Phenotype of the *noot* and *coch* mutant nodules. *M. truncatula* (a) and pea (d) wild-type nodules; *noot* nodules (b, c); *coch* nodules (e, f). The *noot* nodules have increased meristematic activity (b) or show nodule to root conversions (c). The *coch* nodules in (e and f) have multiple roots developing in apical position. The mutant roots originating from the nodule in (f) have root hairs and develop new nodules (asterisk).

49.2 THE NODULE ROOT ORIGINATES FROM NODULE VASCULATURE

The specificity of the *noot* and *coch* mutants among the plant symbiotic mutants is the formation of one to four roots in an apical position of the symbiotic organ. These are genuine roots as they can develop root hairs (Fig. 49.2f) and can themselves bear LR and nodules (Fig. 49.2f). As both mutant roots and nodules are functional, we do not understand at the moment how the plant solves the sink–source functioning in these Russian doll-like structures (root nodule–root nodule). Our work has shown that the roots do not originate from the meristem but are in continuity with the peripheral vascular tissue of the symbiotic organ (Couzigou et al., 2012). This relation to the vascular tissue explains the presence of multiple roots for some nodules (Fig. 49.2c, 2e, and 2f). The

development of these roots can result from the reactivation of the vascular tissue initials present in apical position of these tissues (Fig. 49.1; Osipova et al., 2012). This suggests that one role for NOOT (or COCH) is to suppress the activity of these initials, and that they in fact correspond to silent RAMs. This hypothesis is in agreement with the pattern of expression of the RAM marker *WOX5* and *DR5-RFP* in apical position of the vascular tissue in indeterminate nodules (Osipova et al., 2012; Couzigou et al., 2013).

All these results thus indicate that each nodule vascular element can be considered ontologically homologous to a root. As two vascular traces are detected at early stages of nodule formation (Guinel, 2009), the symbiotic organ is thus initially made of two modified roots surrounding a cortical (symbiotic) tissue. During later steps of nodule development, vascular bundles dichotomize into multiple peripheral vascular tissues.

The conversion from a nodule to a root-like organ can be observed at different stages of the symbiotic organ development as hybrid organs can be formed from very early stages of nodule development to late stages corresponding to mature nitrogen-fixing organs. Conversion from early stages can be sometimes detected only by the presence of a double vascular tissue at the base of the root (Couzigou et al., 2012). When using a GFP-tagged *Rhizobium* strain, discrete foci of infected tissue can be detected at the base of these roots. All intermediates between these early converted roots to mature nodules with one apically developed *noot*-root can be found in the same experiment. This observation suggests that NOOT is acting during the whole nodule organogenesis to maintain the symbiotic organ identity. It remains to determine if its action start after the primordium formation or is already necessary to delimit the primordium territory in the root (see next paragraph). One additional observation is that the penetrance of the mutation is variable in one plant and in between plants species because the *coch* mutants generally develop hybrid organs (Ferguson et al., 2010) in 90% of the nodules in mutant plants, when hybrid structures are generally observed in only 20–40% of the nodules in Medicago *noot* plants (Couzigou et al., 2012). These differences may be explained by the different genetic contexts, resulting in different regulatory mechanisms or may suggest the presence of other actors with similar roles in these plants.

49.3 IS NOOT NECESSARY TO DELIMIT DOMAINS IN THE SYMBIOTIC ORGAN?

In addition to developing a root in nodule apical position, the *noot* and *coch* mutants are characterized by nodules with significantly enlarged meristematic region and increased lobe number (Fig. 49.2b; Ferguson and Reid, 2005; Couzigou et al., 2012). Spots of anisotropic cell divisions are often observed in the meristematic region of these mutant nodules (Fig. 49.3a–c). Some proliferative nodules are also characterized by a callus-like texture in the *noot* and *coch* mutants. *NOOT* and *COCH* are thus necessary for the harmonious growth of the indeterminate nodules, and this suggests an impaired balance between proliferation and differentiation in the mutant backgrounds.

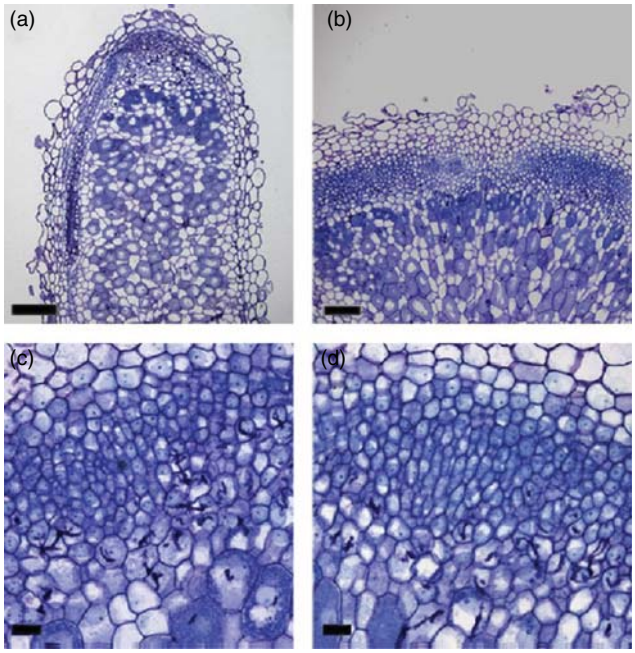

Figure 49.3 Increased meristematic activity in *noot*-mutant nodules. The Technovit section in (a) shows the structure of a wild-type elongated nodule with a small meristem in apical position. The Technovit section in (b and c) shows the meristematic region in a *noot* nodule similar to Fig. 49.2b. The meristem region is enlarged (b) and shows anisotropic cell divisions (c). Scale bar 250 µm in (a and b) and 50 µm in (c).

Plant hormones play an important role in regulating the ratio between division and differentiation in meristems. Auxin and cytokinin were shown to play such roles during nodule formation (Op den Camp et al., 2011b, Deinum et al., 2012, for review see Desbrosses and Stougaard, 2011; Oldroyd et al., 2011; Chapter 56 and Table 49.1) and auxin and cytokinin responsive genes are induced in the nodule meristem (Plet et al., 2011; Couzigou et al., 2013), suggesting that these two hormones might be regulating the nodule meristem activity antagonistically as for the primary meristems (see Chapters 47, 56, 66). Moreover, similar roles for auxin and cytokinin were described for the development of the determinate *Lotus japonicus* nodules (Takanashi et al., 2011; Suzaki et al., 2012), suggesting that part of

the auxin–cytokinin-dependent organogenesis program is conserved between the two types of nodules. The indeterminate nodules of the cytokinin-insensitive *cre1* mutant in *M. truncatula* are characterized by an increased lobe number as we observed in the *noot* and *coch* mutants (Plet et al., 2011). Similarly, an auxin overproducing strain of *Rhizobium leguminosarum* bv. *viciae* induces vetch nodules with an enlarged and more active meristem (Camerini et al., 2008). These results might suggest an interaction between NOOT and the hormone-signaling pathways. However, expression studies (http://mtgea.noble.org, Benedito et al., 2008) do not suggest a direct regulation of *NOOT* expression by auxin or cytokinin. In addition, root elongation is not altered by hormone application in the *noot* mutant (Couzigou et al., 2012).

By contrast, it was shown that the *Arabidopsis BOP* genes are necessary to set boundaries in lateral organs in order to control their development (Ha et al., 2007; Barton, 2010). We can thus hypothesize that *NOOT* and *COCH* fulfill similar functions in boundaries, definitions that will delimit territories and restrict the apical meristem size in the symbiotic organ. The similarities in the *noot* and *bop1bop2* phenotypes in the aerial part of the corresponding mutants (flower and leaf identity) are in agreement with this hypothesis. For example, the *BOP* and *PUCHI* genes are together necessary for bract suppression (Karim et al., 2009), in agreement with the modified and elongated bracts present in *coch* flowers (Gourlay, 1999; Yaxley et al., 2001; Couzigou et al., 2012). In addition, BOP and PUCHI by promoting the expressions of *LEAFY* (*LFY*) and *APETALA1* (*AP1*) (Karim et al., 2009) participate together in the identity of the floral meristem. Similarly, the pea *LFY* ortholog, *UNIFOLIATA* interacts genetically with *COCH* for stipule, flower, and inflorescence development (Gourlay et al., 2000; Kumar et al., 2011; Sharma et al., 2012). These observations thus suggest orthologous functions for the NBCLs present in the different plants.

It remains to determine if the *NBCL* genes play roles during root development. Such role was not described for *BOP* genes, and we did not observe an effect of the *noot* mutation on the root structure but subtle effects cannot be excluded. Using the hypothesis of the orthologous function, it is interesting to note that the *AP2/EREBP* auxin-responsive gene *PUCHI* that interacts genetically with the *BOP* genes (Karim et al., 2009) is required to restrict the zone of proliferation in the lateral root primordia (Hirota et al., 2007). Kang et al. (2013) recently proposed a concerted action of PUCHI with LBD16/ASL18 and LBD18/ASL20 to control lateral root primordium development and size, as well as lateral root emergence, supporting a role for these factors in territory definition. Assuming the functional orthology hypothesis, these results make *PUCHI-LIKE* genes in legumes possible interacting partners. Studying *PUCHI-LIKE* genes expression patterns during nodule and root formation might also be interesting as PUCHI is an early marker for root primordium formation functioning downstream auxin signaling.

49.4 NOOT-LIKE STRUCTURES CAN BE TRIGGERED BY ENVIRONMENT OR MUTANT RHIZOBIA

The formation of roots from indeterminate and determinate nodules (i.e., with or without persistent apical meristem, respectively) was previously reported (Table 49.1). This includes emergence of ectopic roots on (i) *M. sativa* and Trifolium nodules as a result of increased temperature (Day and Dart, 1971; Dart, 1977), (ii) Arachis nodules formed on chimeric plants produced by hairy roots (Akasaka et al., 1998; Sinharoy and DasGupta, 2009), or (iii) after inoculation of Phaseolus, Trifolium, or Soybean plants with *Rhizobium etli*, *R. leguminosarum* bv. *trifolii*, and *Bradyrhizobium japonicum* mutant strains, respectively (McIver et al., 1997; Ferraioli et al., 2004; Morris and Djordjevic, 2006; Gourion et al., 2009). The existence of *noot*-like phenotypes in determinate nodules shows that the conversion of a nodule into a root can be independent of the presence of a persistent meristem in agreement with the vasculature apical origin of the ectopic roots in *noot*. Interestingly enough, these experiments indicate that a bacterial mutant can also induce *noot*-like structure, suggesting a role for the bacterial partner in maintaining the organ identity. The signaling mechanisms underlying the loss of organ identity in presence of a bacterial mutant is still unknown.

It should be noted here that the origin of the root was not precisely determined in all the studies described earlier. Nodules with one central vascular tissue can also be found in some *M. truncatula* mutants or in nodules induced by bacterial mutants (Guan et al., 2013). These hybrid structures are more related to infected roots than to *noot* nodules and seem to be induced after early aborted infections. They may correspond to earlier alterations of the symbiotic organ development, as the vasculature initiation is not altered in *noot*. In agreement with this, a *Rhizobium leguminosarum* purine auxotroph strain induces nodules with a central vasculature in Phaseolus (Newman et al., 1992). If the mutant strain is supplemented with 4-aminoimidazole-5-carboxamide (AICA) riboside for at least 6 days, the nodules have peripheral vascular tissues. These mutant nodules with central vasculature are more similar to the primitive actinorhizal nodules and to rhizobia-induced nodules on the nonlegume *Parasponia andersonii* (Gualtieri and Bisseling, 2000; see Chapter 4). Together with the *noot* (or *coch*) mutant, they will be instrumental in future work to better understand nodule evolution.

Table 49.1 Modified nodules or nodule-like structures described in the literature

Nodule Modification		Bacteria	Host	Root/Nodule Structure	References
Ectopic root formation					
Bacterial mutant	ΔphyR, ΔecfG	*B. japonicum USDA110*	*V. radiata* and *G. max*	Distal root	Gourion et al. (2009)
	lysA, *fbaB*, *purF*, *pyrB*, and *pcka* metabolic mutants	*R. elti*	*P. vulgaris*	Distal root, connected to vasculature	Ferraioli et al. (2004)
	CE106 *Tn5* mutant	*R. phaseoli*	*P. vulgaris*	Central vasculature (ectopic root face to vasculature, not shown)	Vandenbosch et al. (1985), Newman et al. (1992)
	JT205 *Tn5* mutant	*S. meliloti*	*M. sativa*	Pseudonodules with central vasculature	Dudley et al. (1987)
Plant mutant	*noot/coch*	*S. meliloti*, *R. leguminosarum*	*M. truncatula / P. sativum*	Distal root, connected to vasculature	Couzigou et al. (2012), Voroshilova et al. (2003)
Increased temperature		–	*M. sativa, T. repens*		Dart (1977), Day and Dart (1971)
Hairy root transformation	DMI3 RNAi	*Bradyrhizobium* sp. *A2R1*	*A. hypogea*	Root connected to vasculature	Akasaka et al. (1998)
			A. hypogea		Sinharoy and DasGupta, 2009
Hybrid structure/morphological alteration					
Plant mutant	*vapyrin, lin4*	*S. meliloti*	*M. truncatula*	Infected nodule, central vasculature	Guan et al. (2013)
	cre1	*S. meliloti*	*M. truncatula*	Increased lobe number	Plet et al. (2011)
	snf2	*M. loti*	*L. japonicus*	Spontaneous nodule formation	Tirichine et al. (2007)
	hit1	*M. loti*	*L. japonicus*	Infection +/organogenesis −	Murray et al. (2007)
	latd/nip	*S. meliloti*	*M. truncatula*	Nodule meristem arrested	Veereshlingam et al. (2004), Bright et al. (2005), Yendrek et al. (2010)
	hap2/nfya1	*S. meliloti*	*M. truncatula*	Nodule meristem arrested	Combier et al. (2006)
Bacterial mutants	Mutant strain 3160	*B. japonicum*	*G. max*	Infected nodule, central vasculature	Yang et al. (1993)
	exoY	*S. meliloti*	*M. truncatula*	Infected nodule, central vasculature	Guan et al. (2013)
		R. leguminosarum bv. *trifolii*	*T. repens*	Hybrid structure, central vasculature	McIver et al. (1997)
		S. meliloti	*M. sativa*	Hybrid structure, central vasculature	Truchet et al. (1985)
		A. tumefasciens and *E. coli* carrying *Nod* genes	*M. sativa*	Pseudonodule formation	Hirsch et al. (1984, 1985)

(*continued*)

Table 49.1 (*Continued*)

Nodule Modification		Bacteria	Host	Root/Nodule Structure	References
Phytohormone manipulation	Auxin overproducing rhizobia	—	*V. sativa*	Increased lobe number and meristem activity	Camerini et al. (2008)
	Auxin transport inhibitors	—	*M. truncatula*	Pseudonodule formation	Rightmyer and Long (2011)
	Auxin transport inhibitors	—	*M. sativa*	Pseudonodule formation	Scheres et al. (1992)
	Auxin transport inhibitors	—	*M. sativa*	Pseudonodule formation	Hirsch et al. (1989)
	Auxin transport inhibitors	—	6 legume species	Pseudonodule formation	Allen et al. (1953)
	Auxin transport inhibitors	—	*M. sativa*	Pseudonodule formation	van de Wiel et al. (1990)
	Synthetic auxin analog	Increased following rhizobial innoculation	*T. aestivum*	Pseudonodule formation	Fransisco et al. (1994)
	Auxin transport inhibitors	—	*O. sativa*	Pseudonodule formation	Ridge et al. (1993)
	Constitutive trans-zeatin secreting strains (rhizo + coli)	—	*M. sativa*	Pseudonodule formation	Cooper and Long (1994)
	Kinetin application	—	*N. tabaccum*	Pseudonodule formation	Arora et al. (1959)

49.5 ARE NOOT AND COCH INVOLVED IN NODULE IMMUNITY?

A recent study by Canet et al. (2012) suggests that the *Arabidopsis* BOP proteins can interfere with plant immunity by the interaction with TGA bZIP transcription factors. These TGA transcription factors usually control plant immunity by the interaction with the NPR1 protein that belongs to the group of BTB–POZ ankyrin domain proteins (Canet et al., 2012) including BOPs, COCH, and NOOT.

It will thus be interesting to test the role of the *NOOT* gene in immunity against root and foliar pathogens. In addition, our laboratory has recently shown that immunity might be actively repressed in the symbiotic organ in order to allow an efficient bacterial infection of the symbiotic cells (chronic infection; see Bourcy et al., 2013a,b). NOOT may participate to this process by interacting with a NPR1-like dependent signaling pathway as described by Canet et al. (2012). However, the symbiotic bacterial recognition may also require that the symbiotic cells in the nodule have acquired a NOOT-dependent "symbiotic identity." In other words, a root-like identity of the symbiotic organ may not allow the proper recognition of the bacteria and trigger some defense-like reactions in the invaded cells. In agreement with this hypothesis, some defense-like reactions (phenol

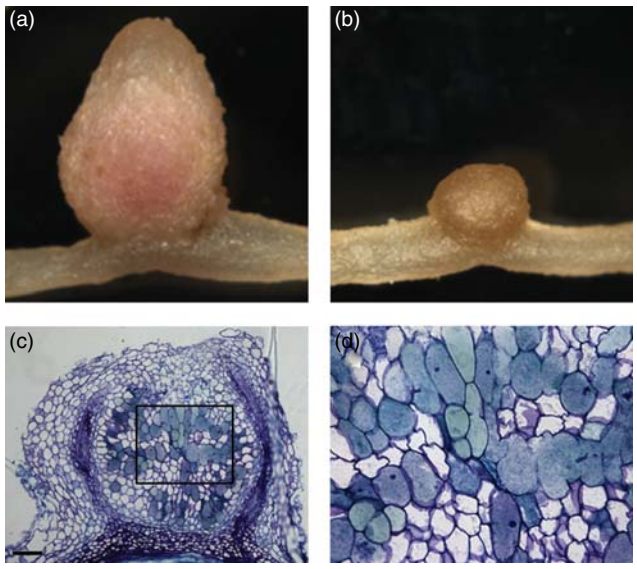

Figure 49.4 Defense-like reactions of the *noot*-mutant nodules. Wild-type nodule (a) and a *noot* nodule showing defense-like symptoms in (b). The toluidine blue stained Technovit section in (c) shows the presence of empty cells that are filled with green material characteristic of phenol accumulation with this staining. (d) Enlargement of a Technovit section showing a symbiotic nodule region showing phenol accumulation. Scale bar 100 µm in (c).

accumulation) were observed sporadically in the *noot* and *coch* mutant backgrounds (Fig. 49.4a–d). Accordingly, these abortive dark nodules show an endoreduplication index more closely related to roots than to wild-type and *noot*-fixing nodules (not shown), probably reflecting an impaired bacteroid differentiation. Future work will be necessary to better define this possible subtle role of NOOT and COCH.

49.6 CONCLUSIONS

Our work (Couzigou et al., 2012) showed that the *NOOT* and *COCH* genes are necessary for the robust persistence of nodule identity. *NOOT* and *COCH* are *Arabidopsis BOP* orthologs, and their functions in the identity of the lateral aerial organs are conserved in *M. truncatula* and *P. sativum* (Hepworth et al., 2005; Norberg et al., 2005; Couzigou et al., 2012). This suggests that *NOOT* and *COCH* were recruited from the aerial developmental programs to repress root identity in the legume symbiotic organ, even if a role in roots cannot be formally excluded. In *Arabidopsis*, the BOP proteins are expressed at the tissue boundaries to define the organ identity and appear to act within the leaf to affect development at the SAM (Barton, 2010). Similar to its *Arabidopsis* orthologs, the *NOOT* expression pattern (Couzigou et al., 2012) suggests a function in the delimitation of the boundaries between the nodule meristem and the vascular tissue initials in the symbiotic organ. In the *noot* and *coch* mutants, the ectopic roots do not derive from the nodule meristem but originate from the vascular initials (Fig. 49.1; Couzigou et al., 2012). This suggests that the nodule vascular strands are ontologically related to roots in agreement with the root-like nature of the nodule vasculature as shown by Osipova et al. (2012) using the WOX5 root identity marker. Thus, similar to their role in the definition of boundaries in Arabidopsis, *NOOT* and *COCH* expressions in the vascular initials may affect the functioning of the nodule meristem and/or regulate the activities of vasculature initials to a level compatible with the coordinated development of these vascular tissues and the nodule meristem.

49.7 PERSPECTIVES

The characterization of the *noot* and *coch* mutants and the identification of the corresponding genes as orthologs of the *BOP* genes allow us to better understand the organogenesis of the temperate legume nodules and may also shed light on their origin(s). However, the known multiple roles of the *Arabidopsis* BOP in interaction with its molecular partners (transcription factors) in root, flower, and leaves do not favor one nodule origin, that is, are they of shoot or root origin (Hirsch and Larue, 1997) even if the phenotype of the mutants point to a root origin of the vascular tissues. Future work will have to identify the NOOT partners and determine their roles in the identity of the whole symbiotic organ. Understanding how NOOT and COCH define territories inside the nodule is also a challenge but will help understanding the structure of this symbiotic organ and possibly how the bacterial symbiotic partner is confined inside the nodule.

It will also be interesting to determine the role of *NOOT* orthologs in other nodule-forming plants. First, we can predict a role for *NOOT* orthologs in determinate nodules as the vascular tissue formation should be similar in these organs (Guinel, 2009), and the nodular roots formed in such nodules are closely linked to vasculature (see references in Table 49.1). The nodules formed in actinorhizal plants and Parasponia have a central vasculature considered as a modified root (Fig. 49.1) and could represent a more ancestral state of the legume symbiotic organ (Gualtieri and Bisseling, 2000). It has been recently proposed that the Parasponia–Rhizobium symbiosis could correspond to a delicate balance between mutualism and parasitism (Op den Camp et al., 2012). The role of *NOOT* orthologs in the identity of these more ancestral symbiotic structures will indicate if the NBCL clade was recruited early for symbiosis or if they are only important for nodules with peripheral vasculature.

The possible complexity of the symbiotic organ with root-like vascular tissue together with shoot-like apical meristem and symbiotic tissue will make these studies challenging but illustrate well the evolutionary tinkering used to recruit pre-existing developmental programs in order to construct new organs (Jacob, 1977).

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Section 9

Recognition in Nodulation

Chapter 50

Flavonoids Play Multiple Roles in Symbiotic Root–Rhizosphere Interactions

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50.1 INTRODUCTION

Flavonoids are plant metabolites that are synthesized through the phenylpropanoid pathway. The precursors of the flavonoid pathway, p-coumaroyl-CoA and malonyl-CoA, can be assembled into 10,000 or more diverse flavonoid molecules in plants (Winkel-Shirley, 2001). Flavonoids generally contain two phenyl rings and a three-carbon heterocyclic ring, arranged in different basic structures (Fig. 50.1). However, the diversity of these molecules is largely contributed by modifications to these basal structures such as through glycosylation, methylation, hydroxylation, acylation, malonylation, prenylation, or polymerization (Winkel-Shirley, 2001). The modifications of the flavonoid structures lead to changes in their physical, chemical, and biological properties such as their solubility, mobility across membranes, degradation by rhizosphere organisms, or their functional properties inside the plant or in the rhizosphere.

The biosynthesis of flavonoids has been part of many studies and the majority of the enzymes involved in the processes have been identified (Dixon and Steele, 1999; Winkel-Shirley, 2001). Flavonoid synthesis is thought to initiate at enzyme complexes located on the cytosolic side of the endoplasmic reticulum in plant cells (Jorgensen et al., 2005). The intermediate structures are then transported into the vacuole for storage and are often glycosylated in the process by enzyme complexes on the tonoplast (Aoki et al., 2000; Winkel, 2004).

Inside plant cells, flavonoids have been localized to the cytoplasm, the vacuole, the nucleus, cell walls or cell membranes (Erlejman et al., 2004; Hutzler et al., 1998; Naoumkina and Dixon, 2008; Saslowsky et al., 2005). The differential localization and synthesis of flavonoids is regulated by several transcription factors belonging to the myeloblastosis (MYB) or basic helix-loop-helix (bHLH) families (Verdier et al., 2012). These transcription factors are able to respond to multiple environmental cues (Koes et al., 2005).

Flavonoids can be moved between cells and into the rhizosphere through various transporters, although their function and specificity still remains to be elucidated. It is likely that the cell-to-cell transport of flavonoids occurs through a vesicle-mediated transport or through membrane-bound transporters such as MATE (multidrug and toxin extrusion protein) and ABC (ATP binding cassette)-type transporters (Zhao and Dixon, 2009). The transport of flavonoids also occurs over long distances, as the external application of flavonoids in *Arabidopsis* causes them to move to the more distal parts of the plant (Buer et al., 2007). The mechanism of this transport is thought to be mediated through the members of ABC transporter families or through glutathione complexing.

To reach potential symbionts in the rhizosphere, flavonoids are either actively exuded by the root through transporters (Badri et al., 2008, 2012; Sugiyama et al., 2008)

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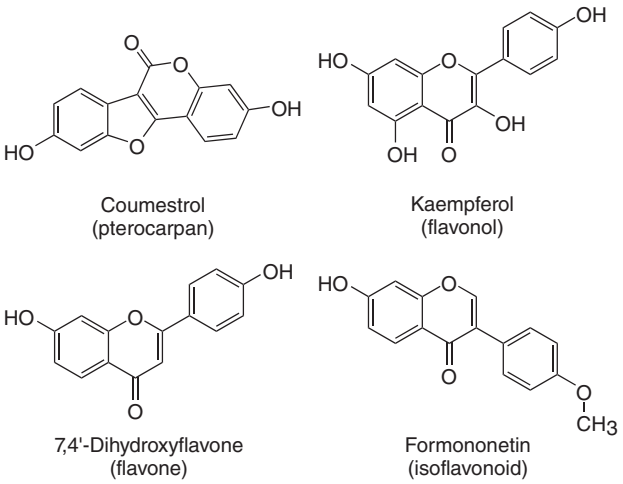

Figure 50.1 Examples of flavonoid structures with roles in root–symbiont interactions.

or released from root border and root cap cells (Hawes et al., 1998; Shaw et al., 2006). The synthesis and exudation of flavonoids are also highly responsive to the presence of biotic elicitors and this is likely to result in fine-tuning of the signaling interaction between plant hosts and symbionts as well as pathogens (Armero et al., 2001; Schmidt et al., 1994).

50.2 ROLES FOR FLAVONOIDS IN THE *RHIZOBIUM*-LEGUME SYMBIOSIS

Most legumes have the ability to form a symbiotic relationship with nitrogen-fixing bacteria collectively known as rhizobia.

The symbiosis requires partner recognition and specificity, which are partially mediated by plant-exuded flavonoids. Nodulation also involves the development of root nodules from root cortical and pericycle cells, which is controlled in part through polar auxin transport and auxin accumulation. Flavonoids control auxin transport and breakdown during nodule development. Thus, flavonoids have roles in the rhizosphere as signals to rhizobia, and in addition act inside the root to control nodule development, as described in the following text (Subramanian et al., 2007).

Flavonoids act in several ways during symbiosis, such as chemically attracting rhizobia, inducing *nod* gene synthesis in rhizobia, acting as determinants of host specificity, as regulators of root development, or by altering plant defence responses (Cooper, 2004).

The regulation of *nod* genes in rhizobia by root-secreted flavonoids has been well studied. The *nod* genes encode enzymes that synthesize species-specific lipochitooligosaccharide molecules known as Nod factors. The Nod factors are required for the formation of nodules and the invasion of rhizobia in the host plant (Spaink, 1995; see Chapter 51). Nod genes are regulated by NodD transcription factors that belong to the LysR family of transcriptional regulators. When an appropriate flavonoid binds to the NodD protein it improves the access of RNA polymerase to the *nod* gene promoter, thereby increasing its transcription (Li et al., 2008; Peck et al., 2006). The transcription factor NodD is also activated by an increase in the concentration of intracellular calcium induced by flavonoids (Moscatiello et al., 2010). The *nod* gene inducing flavones luteolin, isolated from *Medicago sativa,* and 7,4′-dihydroxyflavone (Fig. 50.1) from *Trifolium repens* (Peters et al., 1986; Redmond et al., 1986) were the first flavonoid molecules shown to have an effect. This *nod* gene inducing property has since been discovered for many other flavonoids, although some also function as *nod* gene repressors, as summarized by Cooper (2004). It is thought that a combination of flavonoids is more effective as *nod* gene inducers rather than a single compound (Begum et al., 2001; Bolanos-Vasquez and Warner, 1997). This combination of flavonoids as perceived by the rhizobia is often species-specific and determines which rhizobia are able to successfully establish a symbiotic relationship with the host plant. The affinity of the NodD protein to flavonoids partially determines host range, and it has been shown that NodD proteins from rhizobia with a broad host range, for example, *Rhizobium* sp. NGR234, are activated by a wider range of flavonoids than NodD from narrow host range rhizobia (Gyorgypal et al., 1991; see Chapter 32).

As mentioned previously, not all flavonoids are *nod* gene activators. For example, the isoflavonoids medicarpin and coumesterol (Fig. 50.1) have been shown to negatively regulate *nod* gene expression in *Sinorhizobium meliloti* (Zuanazzi et al., 1998). Together, the combinations of flavonoids maintain an optimal level of Nod factor secretion while also preventing elicitation of defence response by the plant (Savouré et al., 1997; Zuanazzi et al., 1998).

The flavonoid secretion profile from a host root changes during the course of a symbiotic relationship with rhizobia. The flavonoid exudates from different legume species show variation in their composition after inoculation with rhizobia. It is likely that this effect is important to optimize the synthesis of Nod factors during different stages of symbioses. For example, the plant does not secrete high amounts of *nod* gene activating flavonoids after it has formed sufficient numbers of nodules (Dakora et al., 1993; Schmidt et al., 1994). The ability of flavonoids to be metabolized and modified by rhizobia also affects their activity as *nod* gene inducers or inhibitors (Rao and Cooper, 1995).

In addition to *nod* gene activation of their specific symbionts, flavonoid exudation from the root also plays a role in guiding rhizobia to a host root. Some rhizobia display strong chemo-attraction to root-exuded flavones and flavonones such as luteolin and apigenin. The plant also

displays selectivity in attracting different species of rhizobia with variations in their flavonoid exudation profile (Aguilar et al., 1988; Dharmatilake and Bauer, 1992).

The exudation of flavonoids from legume roots can be modified not only in response to rhizobia, but also free-living nitrogen-fixing bacteria. For example, a study by Star et al. (2012) found that co-inoculation of *Vicia sativa* spp. *nigra* with its symbiont *Rhizobium leguminosarum* bv. *viciae* and the free-living nitrogen-fixing bacterium *Azospirillum brasiliense* enhanced flavonoid exudation, and this was dependent on the ability of the *Azospirillum* strain to synthesize auxin (see Chapter 91). Another determinant of flavonoid production is the availability of nitrogen in the growth medium. Flavonoid exudation by legumes remains low under nitrogen-sufficient conditions but is increased in the absence of nitrogen, that is, at a time when the host needs to establish a symbiosis with rhizobia (Coronado et al., 1995).

Legume hosts also exude combinations of flavonoids to select for compatible symbiotic organisms. For example, medicarpin from clover and medic species has an inhibitory effect on incompatible bacterial strains, but does not inhibit the growth of compatible rhizobia (Pankhurst and Biggs, 1980). Low concentrations of isoflavonoids have been reported to induce resistance in compatible symbiotic bacteria to potentially bactericidal phytoalexins from root exudates (Parniske et al., 1991). This suggests additional sites of recognition apart from a *nodD* promoter site and allows the plants to protect the beneficial bacteria while mounting a defence against pathogenic organisms.

Some of the other flavonoid-regulated genes known in rhizobia include genes for exopolysaccharide synthesis (see Chapter 36). The exopolysaccharide layer in rhizobia is important for the regulation of defence response in the host plant (Dunn et al., 1992). Some exuded flavonoids, for example, daidzein and genistein, have been implicated in activating the type III secretion system in rhizobia and thus stimulating the secretion of important nodulation outer proteins (Nops) (de Campos et al., 2011; Krishnan et al., 2003).

The perception of *Nod* factors by the root leads to new initiation of cell division in root cortical and pericycle cells, most likely due to auxin accumulation in those cells (Mathesius, 2008; Suzaki et al., 2012). Flavonoids have also been shown to accumulate specifically in cortical cells before and during the onset of cell division (Mathesius et al., 1998a). Flavonoid accumulation is also spatially distinct in meristematic and vascular tissues of differentiated nodules in a host-species specific manner (Charrier et al., 1998). Those studies suggest specific roles of flavonoids in cell division at both the early and later stages of the symbiosis. Some flavonoids such as the flavonol kaempferol (Fig. 50.1) have been found to act as negative regulators of polar auxin transport in clover, mimicking the action of Nod factors on auxin accumulation in the root (Mathesius et al., 1998b; see Chapter 47). Measurements of polar auxin transport showed that acropetal auxin transport is inhibited during infection with rhizobia, and that this inhibition of auxin transport does not occur when the flavonoid pathway is silenced in *Medicago truncatula* (Wasson et al., 2006). Silencing of specific branches of the flavonoid pathway in *M. truncatula* suggests that it is the flavonol branch that is most likely required for auxin transport control (Zhang et al., 2009). Silencing of the isoflavonoid biosynthesis pathway in soybean also leads to changes in auxin responses in the roots, but this can be overcome by inoculation with a genistein-hypersensitive *Bradyrhizobium* strain or with Nod factor addition (Subramanian et al., 2006), suggesting that indeterminate- and determinate-type nodules have different requirements for auxin transport control by flavonoids. Silencing of isoflavone reductase (IFR) in bean also led to reduced nodule numbers, and this was correlated with reduced expression of the auxin response gene *GH3* (Ripodas et al., 2013). In contrast to chalcone synthase silencing in *M. truncatula*, which showed no significant effects on lateral root development (Wasson et al., 2009), silencing of *IFR* in bean reduced root growth and lateral root emergence, which could be related to an effect on auxin (Ripodas et al., 2013).

The exact mechanism through which flavonoids affect the auxin transport is not known, but evidence from *Arabidopsis* suggests that flavonoids can affect the vesicular cycling of auxin transporters of the PIN family, possibly through interactions with other regulatory proteins such as phosphatases and kinases (Peer et al., 2004; Peer and Murphy, 2007; Santelia et al., 2008; see Chapter 47).

The increase or decrease in auxin in dividing cortical cells may also be caused by the regulation of auxin breakdown by peroxidases. Flavonoids again have been implicated in this role as regulators of peroxidase activity. It has been discovered that the isoflavonoid formononetin (Fig. 50.1) from *Trifolium repens* accelerates auxin breakdown through the activation of peroxidases. Other flavonoids such as 7,4′-dihydroxyflavone and its glycosides accumulate in dividing cortical cells where they can inhibit auxin breakdown by peroxidases (Mathesius, 2001). This differential ability of flavonoids and the availability of a large range of such metabolites ensures that the plant can actively regulate the development of new root organs such as nodules.

Flavonoids could also be playing a role in the systemic regulation of nodule numbers. Split-root experiments have shown that the isoflavonoid formononetin and its glycoside ononin are down-regulated in both rhizobia-induced and mycorrhiza-induced symbioses in a systemic manner, suggesting that a related autoregulation signal affects their synthesis in both processes (Catford et al., 2006). External application of ononin could partially restore the nodulation and mycorrhization of the autoregulated root system, suggesting that flavonoids have an active role in controlling symbiosis (Catford et al., 2006). Similarly, in grafts of

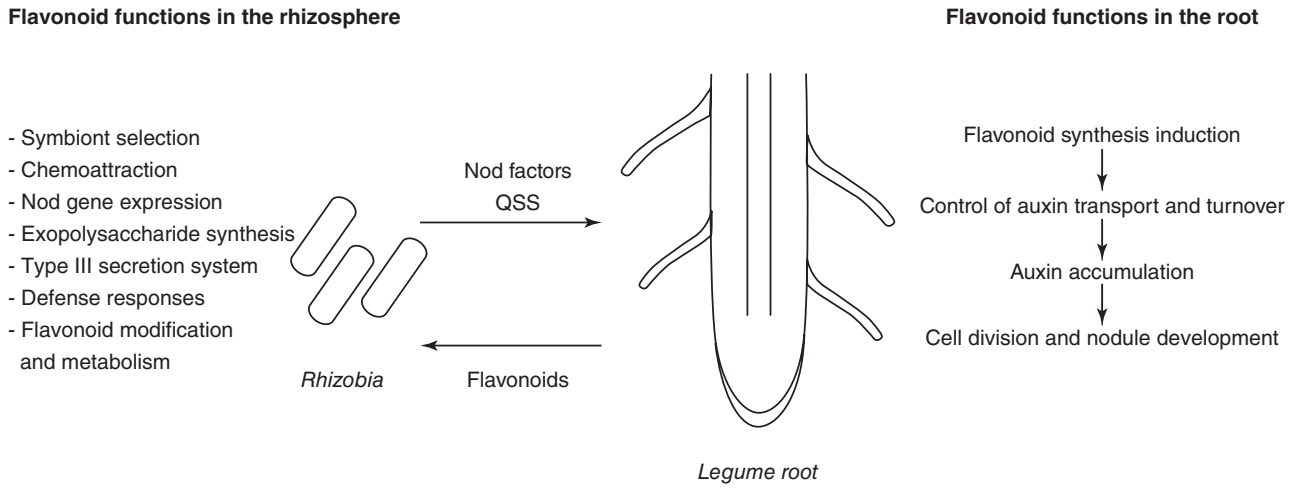

Figure 50.2 Simplified model of the various roles of flavonoids in the *Rhizobium*–legume symbiosis.

supernodulating soybean shoots with wild type bean roots, accumulation of root isoflavonoids is higher in graft combination with a supernodulating shoot than with a wild type shoot (Abd-Alla, 2011). External application of daidzein and coumestrol increases nodule numbers and enhances bacterial growth in culture (Abd-Alla, 2011). It is possible that systemically accumulated flavonoids control auxin transport in the autoregulated roots, as auxin transport has been shown to be modulated during autoregulation in *M. truncatula* (van Noorden et al., 2006). The roles of flavonoids in the *Rhizobium*-legume symbiosis are summarized in Figure 50.2 (see also Chapter 47).

50.3 FLAVONOIDS ARE INVOLVED IN ACTINORHIZAL SYMBIOSES

Apart from the *Rhizobium*-legume symbiosis, nodulation also occurs in several families of non-legumes that form a symbiosis with the actinorhizal bacterium *Frankia*. So far, no *nod* genes have been identified in *Frankia* (Normand et al., 2007) and the early signaling interaction between hosts and *Frankia* are still under investigation (see Chapters 35, 55). There are some indications that flavonoids stimulate the symbiosis. For example, flavonoids present in seed and root exudates have been found to enhance nodulation by *Frankia*, although the mechanism is not understood (Benoit and Berry, 1997; Hughes et al., 1999). Flavonoids might also have a role in bacterial selectivity by the host because the fruits of the plant *Myrica gale* improve the growth and nitrogen fixation ability of the compatible *Frankia* species while repressing incompatible strains (Popovici et al., 2010).

As in legumes, flavonoid biosynthesis genes are differentially expressed during nodulation in actinorhizal plants such as *Casuarina glauca*, and some of the transcripts are up-regulated in the absence of an external nitrogen source (Auguy et al., 2011). Flavans have been found to accumulate in specific compartments of the nodule lobes of actinorhizal plants, where they have been hypothesized to restrict the infection of the symbiont (Laplaze et al., 1999).

50.4 FLAVONOIDS ENHANCE PLANT INTERACTIONS WITH FREE-LIVING NITROGEN-FIXING BACTERIA

Flavonoids appear to play roles not just in legume–rhizobia interactions but also in the colonization of non-legumes with nitrogen-fixing bacteria. Infection of non-legumes with nitrogen-fixing plant-growth promoting bacteria like *Azospirillum* sp. can be host-specific in its effects on plant growth promotion and the induction of flavonoids and other phenolic compounds in the plant (Chamam et al., 2013; see Chapter 90). As in some legumes, for example, *Sesbania rostrata*, which is colonized by *Azorhizobium caulinodans*, infection of non-legumes by rhizobia appears to occur via the so-called crack-entry, mostly at sites of lateral root emergence (Tsien et al., 1983). The colonization of wheat, rice and *Arabidopsis thaliana* by *A. caulinodans* has also been observed around lateral root emergence sites and rhizobia colonize root tissues intercellularly. The frequency of infection is increased in the presence of the *A. caulinodans Nod* gene inducer naringenin, but not with other flavonoids tested (Gough et al., 1997; Jain and Gupta, 2003; Webster et al., 1998). However, this enhanced colonization ability is independent of *nod* gene activation and is unlikely due to the potential of flavonoids to act as a food source for rhizobia in these studies. Interestingly, the colonization of *A. thaliana* by *A. caulinodans* is similar in wild type and flavonoid-deficient mutants, suggesting that plant internal flavonoids are not necessary for the colonization process (O'Callaghan et al.,

2001). While these studies have been carried out under controlled conditions, it has been questioned whether internal colonization of non-legumes with nitrogen-fixing bacteria actually occurs under field conditions and whether the niches occupied by these bacteria in root tissues are viable places for bacterial survival (McCully, 2001). It is currently unknown how externally applied flavonoids could activate colonization of legumes and non-legumes by nitrogen-fixing bacteria, but proteome and transcriptome analysis of rhizobia exposed to flavonoids found multiple protein and transcript accumulation changes, including some related to bacterial stress response, redox control, transport, and biofilm formation (da Silva Batista and Hungria, 2012; de Campos et al., 2011; Guerreiro et al., 1997; Lee et al., 2012). In the endophytic diazotroph *Herbaspirillum seropedicae*, naringenin regulates the synthesis of exopolysaccharides, lipopolysaccharides and auxin, which could be important for regulation of defense responses and developmental changes in the host, for example, lateral root formation by auxin (Tadra-Sfeir et al., 2011; see Chapter 93). Auxin synthesis is also enhanced in the presence of flavonoids in *Rhizobium* sp. NGR234 (Theunis et al., 2004). As naringenin treatment also increases lateral root formation in wheat both in the absence and in the presence of *A. caulinodans* (Webster et al., 1998), it is possible that this effect is via auxin production in the rhizobia or via the interaction of naringenin or downstream flavonoid products with the auxin transport machinery (Wasson et al., 2006).

There is also a possibility that flavonoids such as naringenin interfere with quorum sensing-regulated behaviors in bacteria. Many of the behaviors of bacteria involve cell-to-cell communication signals called quorum sensing signals (QSSs) (Fuqua et al., 2001; see Chapter 37), and this is important for functions including nitrogen fixation and biofilm formation (Gonzalez and Marketon, 2003; von Bodman et al., 2003). Naringenin has been identified as a molecule that interferes with quorum sensing signaling in *Escherichia coli, Vibrio fischeri* (Vikram et al., 2010) and *Pseudomonas aeruginosa* (Vandeputte et al., 2011). However, the effects of naringenin on quorum sensing regulated genes in rhizobia or other rhizosphere bacteria have not been investigated. Flavonoids that induce *nod* gene expression in rhizobia have also been shown to have a stimulatory effect on their QSS synthesis genes (Perez-Montano et al., 2011). Genistein, apigenin, and daidzein have been found to stimulate QSS synthesis, but their activity varies in different species of rhizobia. This suggests that flavonoids could stimulate symbiotic functions in bacteria by influencing their communication.

50.5 ROLES FOR FLAVONOIDS IN MYCORRHIZAL SYMBIOSES

The majority of land plants can be colonized by mycorrhizal fungi that are beneficial contributors in plant phosphorus nutrition. The most studied interaction is the arbuscular mycorrhizal (AM) association. Many of the early signaling steps required for the *Rhizobium*–legume symbiosis are shared in AM symbioses with legumes (see Chapter 108). However, the requirements for flavonoids in nodulation is not shared in mycorrhizal symbioses, as flavonoid-deficient

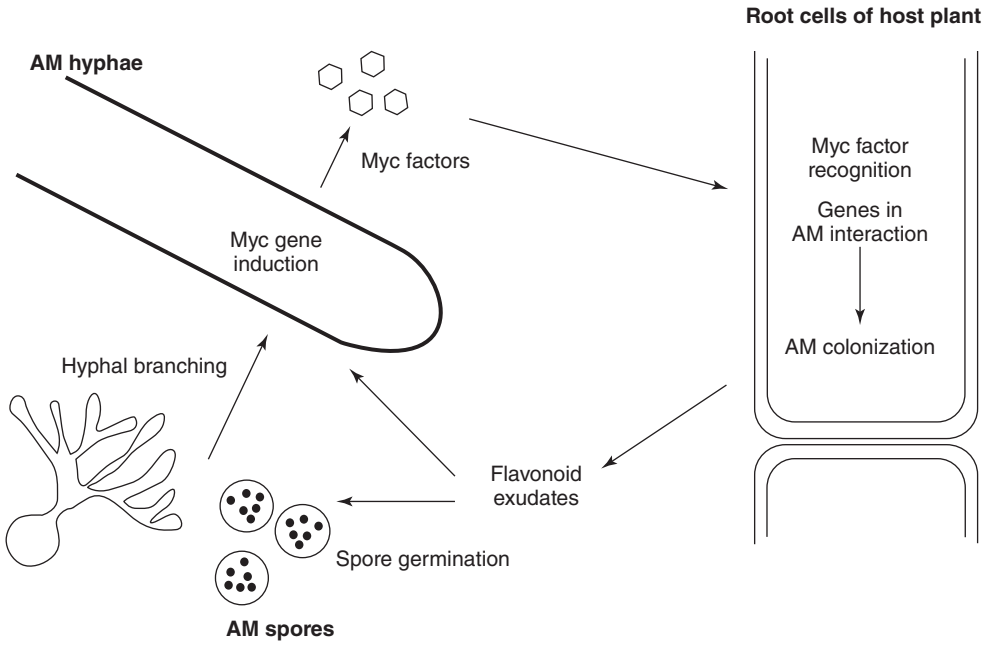

Figure 50.3 Simplified diagram depicting the possible roles for flavonoids in arbuscular mycorrhizal symbioses.

plants have been shown to form functioning AM symbioses (Becard et al., 1995). Nevertheless, flavonoids appear to play stimulating roles that can enhance the symbiosis.

The spores of mycorrhizal fungi germinate in the soil to form hyphae that continue to branch in the presence of specific root exudates (Fig. 50.3). The fungal hyphae are also attracted by the root exudates. As the AM colonization continues, the hyphae form ecto- or endomycorrhizal invasion structures (Harrison, 2005). The flavonoids found in legume root exudates have been reported to stimulate the germination of AM fungal spores, to induce branching of the hyphae, and to regulate the colonization of the roots in a manner that is specific to the symbiont (Kikuchi et al., 2007; Scervino et al., 2005, 2007; Siqueira et al., 1991; Steinkellner et al., 2007). The soil condition also affects the root exudate profile and it has been shown that mycorrhizae-enhancing flavonoids are induced when the plants are deprived of phosphorus in the soil (Akiyama et al., 2002). The isoflavonoid coumesterol (Fig. 50.1) has been identified as an active stimulator of hyphal growth (Morandi et al., 1984). A mutant of *M. truncatula* that overaccumulates coumesterol also showed hyperinfection with its mycorrhizal partner (Morandi et al., 2009).

As the mycorrhizal hyphae invade the root and form arbuscules, the flavonoid profile in the root is altered (Harrison and Dixon, 1994). The flavonoids induced early in this process probably regulate plant defense responses. It has been suggested that there is a temporary up-regulation of flavonoid phytoalexins in the roots in the initial stages, which regulates the invasion of the mycorrhizal fungus. This defense response is used in the later stages as an autoregulation mechanism for the extent of mycorrhizal colonization of the roots (Larose et al., 2002) (Fig. 50.3).

Not all flavonoids may act as stimulators to mycorrhizal growth and colonization. Lupins, when not colonized by AM fungi, secrete pyranoisoflavones that are able to inhibit the branching of mycorrhizal fungi, thereby confirming a duality in the function of flavonoids (Akiyama et al., 2010). Many other host plants also synthesize flavonoids with inhibitory effects on hyphal branching (Tsai and Phillips, 1991). Hence it is possible that host and non-host plants together modify the rhizosphere profile through their root exudates and influence the extent of the symbiotic relationship.

50.6 OUTLOOK

Many of the roles of flavonoids in plant–microbe interactions, particularly between model organisms, have been studied and described in this review. However, most of the interactions have been studied under (semi-) sterile laboratory conditions. Studies under real soil settings will be necessary in the future to determine effective concentrations, stability, and conversions of flavonoids in soil. The ability to modify the flavonoid pathway using RNA interference has proven to be an effective tool for analyzing flavonoid function in legumes and is likely to yield interesting results in future studies in actinorhizal plants.

Further future studies will need to focus on multi-species interactions. The complex nature of the rhizosphere means that the flavonoids exuded by the root may have variable effects on symbionts and pathogens in the soil as well as other plants nearby. Flavonoids may form defence compounds such as phytoalexins or phytoanticipins, in particular against fungal infections (Makoi and Ndakidemi, 2007). Similarly, the chemo-attractant property of flavonoids may attract a number of rhizosphere organisms including symbionts and pathogens. For instance, the isoflavonoid exudates from soybean can attract its compatible symbiont *Bradyrhizobium japonicum* as well as the pathogenic oomycete *Phytophthora sojae* (Morris et al., 1998).

Flavonoids are also able to modify rhizosphere microbial community structures and this is an area that needs further exploration. Some microorganisms are able to use exuded flavonoids as a carbon source and metabolize it. The roots may secrete select flavonoids that these microbes are capable of metabolizing, thus selectively propagating compatible microorganisms. These flavonoids may otherwise act as phytoalexins to inhibit growth of non-compatible microbes (Rao and Cooper, 1994; Walker et al., 2003). An *Arabidopsis* mutant *abcg30* that exhibits higher root phenolics exudation showed extensive changes in bacterial and fungal community structures (Badri et al., 2009). The degradation of flavonoids in the rhizosphere also changes the soil profile and can affect other microbial communities (Shaw et al., 2006). Further studies on the real rhizosphere through techniques such as high throughput sequencing of 16s rRNA to identify bacterial community changes (Peiffer et al., 2013) need to be carried out in order to understand more about the multiple roles flavonoids play in controlling the activities of symbionts and other microbes in the rhizosphere.

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Chapter 51

Nod Factor Recognition in *Medicago truncatula*

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51.1 INTRODUCTION

Nod factors, produced by rhizobia, are essential signaling molecules for establishing the root nodule nitrogen-fixing symbiosis with legumes (Dénarié et al., 1996). Nod factors are lipo-chitooligosaccharides (LCOs) composed of a backbone of generally 3 or 4 β-1,4-linked *N*-acetylglucosamine (GlcNAc) units, which is *N*-acylated on the terminal non-reducing glucosaminyl residue and may be decorated with various chemical substitutions that are important for the specific recognition of the symbiont by its host plant. For example, the major Nod factor of *Sinorhizobium meliloti*, the rhizobial symbiont of *Medicago* plants, contains three GlcNAc units, is O6-sulfated on the reducing residue and the glucosamine unit is *N*-acylated with a C16:2 fatty acid and partially O6-acetylated: it is named NodSm-IV(Ac,S,C16:2Δ2,9) and the presence of the sulfate group is the major determinant of host specificity. The recently discovered Myc-LCOs, which stimulate the agronomically and ecologically important root endosymbiosis with arbuscular mycorrhizal fungi, have a chemical structure very close to that of Nod factors, except that they consist of a mixture of simpler sulfated and non-sulfated LCOs (Maillet et al., 2011).

GlcNAc also constitutes the building block of several microbe-associated molecular patterns (MAMPs) derived from fungal chitin (chitooligosaccharides (COs)) or bacterial peptidoglycan (PGN), which are important signaling molecules in parasite–host interactions (Silipo et al., 2010). In plants, several different proteins/protein domains have been implicated as receptors for such GlcNAc containing signals, including the lysin motif (LysM) (Buist et al., 2008; Gust et al., 2012), present in some extracellular receptor-like proteins (LYMs) and transmembrane receptor-like kinases (LysM-RLKs). For chitin, COs, and PGN, a LysM-RLK (CERK1) and LYM proteins have been shown to bind their GlcNAc-containing signals and to be involved in defense responses (Iizasa et al., 2009; Kaku et al., 2006; Miya et al., 2007; Petutschnig et al., 2010; Wan et al., 2008; Willmann et al., 2011). For Nod factor responses, genetic evidence from various legumes implicates the involvement of two LysM-RLKs (NFP and LYK3 from *Medicago truncatula* and NFR5 and NFR1 from *Lotus japonicus*) (Arrighi et al., 2006; Limpens et al., 2003; Radutoiu et al., 2003; Smit et al., 2007). Recently, high-affinity LCO binding to NFR1 and NFR5 was reported (Broghammer et al., 2012).

In *M. truncatula*, no high-affinity binding has been reported for NFP and LYK3 but biochemical studies, using radiolabeled Nod factors, have characterized Nod factor-binding sites (NFBSs) in *M. truncatula* roots and *Medicago* cell cultures, which differ by their affinity and their selectivity toward the *S. meliloti* Nod factor (Bono et al., 1995; Gressent et al., 1999; Hogg et al., 2006). One of them, NFBS2, has recently been identified and corresponds to the LysM-RLK LYR3 (Fliegmann et al., 2013). In this chapter we will summarize our current knowledge on the molecular mechanisms of Nod factor perception in *M. truncatula*.

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51.2 NFP, A PROTEIN WITH MULTIPLE BIOLOGICAL FUNCTIONS

nfp mutants were first isolated by a genetic screen based on the inability of *M. truncatula* plants to nodulate. Further characterization showed that *NFP* controls all the biological responses triggered by Nod factors during the symbiosis with *S. meliloti* (Ben Amor et al., 2003; Arrighi et al., 2006; El Yahyaoui et al., 2004; Shaw and Long 2003). Both the phenotype of *nfp* mutants and the presence, in the predicted protein, of an extracellular domain with three LysMs known to bind GlcNAc-containing compounds (Bateman and Bycroft 2000), strongly suggested that NFP is the Nod factor receptor. Moreover, complementation experiments of *nfp* mutants using chimeric genes encoding an NFP protein where the LysM domains were replaced by those of PsSYM10, the ortholog of pea, revealed that the second LysM domain of NFP was critical to restore the nodulation and infection processes (Bensmihen et al., 2011; see Chapter 59). Although the extracellular domain of PsSYM10 was expected to interact with non-sulfated Nod factors from *Rhizobium leguminosarum bv viciae*, no change in symbiont specificity was observed, suggesting the presence of an additional component, not yet identified, that is responsible for the perception of sulfated Nod factors in *M. truncatula*.

One of the structural features of NFP is the absence of a catalytically active kinase in the kinase-like intracellular part (Arrighi et al., 2006). Therefore, a ligand-dependent auto-phosphorylation, which is quite a common means for an RLK to activate a signaling pathway, cannot be envisaged for NFP. However, NFP could be trans-phosphorylated by a soluble kinase or another LysM-RLK as reported for NFR5/NFR1 in *L. japonicus* (Madsen et al., 2011). LYK3, a LysM-RLK which possesses an active kinase and which functionally interacts with NFP when expressed in *Nicotiana benthamiana* leaves (Pietraszewska-Bogiel et al., 2013) is an obvious candidate for such a role. However, LYK3 being dispensable for the early responses elicited by Nod factors in the root epidermis, excludes the possibility of an essential role of this protein, at least for this tissue. Alternatively, conformational changes in NFP after ligand binding could result in the activation of the signaling pathway via downstream interacting proteins, which are yet to be identified.

Interestingly, *NFP* is also involved in some of the biological responses elicited by Myc-LCOs, such as lateral root formation, but not in mycorrhization (Maillet et al., 2011). In *Parasponia andersonii*, a non legume plant, the homolog of *NFP* controls both nodulation and mycorrhization (Op den Camp et al., 2011; see Chapter 4). Surprisingly, NFP is also involved in a pathogenic context. Recent studies have reported that, compared to wild-type plants, *nfp* mutants are more susceptible to the root pathogen *Aphanomyces euteiches* (Rey et al., 2013), which causes severe damage in legume crops (Gaulin et al., 2007), as well as to the fungus *Verticilium albo-atrum* (Ben et al., 2013). It could be hypothesized that NFP is involved in the recognition of GlcNAc compounds released from the cell wall of these microorganisms and acting as MAMPs. Indeed, *A. euteiches*, which belongs to the Saprolegniale, contains GlcNAc-based cell wall components organized in a non-crystalline structure (Badreddine et al., 2008) and recently identified as chitosaccharides linked to β-1,6-glucans (Nars et al., 2013). However, the chitosaccharide-enriched fraction, obtained from the *A. euteiches* cell wall, exhibited similar biological effects, in terms of induction of nuclear calcium transients and expression of defence genes, on roots of *M. truncatula* wild-type plants and *nfp* mutants, suggesting a role of NFP in immunity rather than in MAMP perception.

The multi-faceted biological role of NFP questions its biochemical function. The working model proposed by Gough and Jacquet (2013) hypothesized that NFP could participate in different receptor complexes: the perception of a given signal would depend on the interacting partner of NFP within the receptor complex that would lead to the activation of the signaling pathway and the appropriate biological response. This model is reminiscent of the functioning of AtCERK1, the LysM-RLK involved in chitin perception, which, together with the LysM proteins LYM1 and LYM3, participates in PGN perception. If this model is correct it would explain why, despite many efforts and attempts by various approaches, no high-affinity binding to Nod factor has been found for NFP (JJ. Bono et al., unpublished data).

51.3 NOD FACTOR BINDING SITES ARE INDEPENDENT OF NFP

The biochemical approach developed to identify putative Nod factor receptors was based on the use of a radioactive Nod factor to perform ligand-binding experiments on plant extracts. A tritiated Nod factor was first obtained by total chemical synthesis (Bono et al., 1995). Because the specific radioactivity of tritium was relatively low and did not enable the detection of high-affinity binding proteins, a procedure to label the Nod factor to a higher specific radioactivity using ^{35}S was established (Bourdineaud et al., 1995; Gressent et al., 2004). In all cases the chemical structure of the radioactive Nod factor was identical to that of the genuine Nod factor produced by *S. meliloti*. The biochemical approach has allowed the characterization of three NFBSs. Their characteristics are reported in Table 51.1.

All the NFBSs recognize both sulfated and non-sulfated Nod factors with the same affinity and all are independent of *NFP*. NFBS1 and NFBS3 are associated to a high-density root fraction of *M. truncatula*. Because the use of fluorescent derivatives of Nod factors showed that they

Table 51.1 Sub-cellular localization and binding characteristics of the Nod factor binding sites NFBS1, NFBS2, and NFBS3, in *M. truncatula*.

	Low affinity		High affinity	
	NFBS1		NFBS2	NFBS3
	Root	Cell culture	Cell culture	Root
Sub-cellular fraction	3000xg fraction	Microsomal fraction	Microsomal fraction	3000xg fraction
*Affinity (K_d, in nM)	86	107	4	0.45
Abundance (B_{max}, in pmol/mg protein)	2	1	0.5	0.25

*Measured for NodSm-IV(Ac,S,C16:2Δ2,9).

accumulate in the cell wall of root hairs, it is tempting to suggest that these binding sites could be associated to the cell wall compartment (Goedhart et al., 2003). NFBS3 differs from NFBS1 by its affinity for the NodSm factor (K_d of 0.45 nM vs 86 nM for NFBS1) and by the fact that it is not detected in *dmi1* and *dmi2* mutants (Hogg et al., 2006). As DMI1 and DMI2 are respectively located near the nuclear envelope (Riely et al., 2007) and the plasmamembrane or the cytosolic vesicles after Nod factor treatment (Riely et al., 2013), the lack of NFBS3 in *dmi1* and *dmi2* mutants could be due to an indirect effect of these mutations rather than a direct role of DMI1 and DMI2 proteins in the binding site.

NFBS2 was first characterized in cell suspension cultures of *M. varia* and *P. vulgaris* (Gressent et al., 1999, 2002). NFBS2 displays interesting characteristics in terms of Nod factor recognition: it exhibits a high affinity for the Nod factor, it specifically recognizes the lipo-chitoligosaccharidic structure as do NFBS1 and NFBS3 but it differs from these binding sites by its ability to discriminate the length of the chitin backbone and of the fatty acid, which are important for the biological activity of the Nod factors (Demont-Caulet et al., 1999). However, it does not specifically recognize the sulfate group. Nevertheless, it was challenging to identify the protein(s) responsible for such a binding site which, moreover, did not correspond to NFP.

51.4 THE LYSM-RLK LYR3: A NEW PLAYER IN NOD FACTOR PERCEPTION?

In *M. truncatula*, a site similar to NFBS2 was found to be more abundant (at least 5 times) in a cell culture line generated from the roots of a *dmi3* mutant compared to a wild-type line (Hogg et al., 2006). This difference of abundance between the two cell lines offered the opportunity to identify the binding protein by combining photoaffinity labeling experiments with proteomic and transcriptomic approaches (Fliegmann et al., 2013). For that purpose, photoactivatable derivatives of Nod factors, based on the structure of aromatic LCO analogs that tightly bind to NFBS2 (Grenouillat et al., 2004), were first synthesized. The photoreactive group, either an azido or a benzophenone group, was positioned in the vicinity of the non-reducing unit of the oligosaccharidic chain in order to increase the chance to specifically crosslink the binding protein, because binding studies had shown that this part of the molecule was involved in the interaction with NFBS2. Photoaffinity labeling experiments reported in Figure 51.1 enabled to detect a polypeptide specifically crosslinked to the Nod factor derivative for the *dmi3* sample but not for the wild-type, with an apparent molecular mass close to 100 kDa, thus in accordance to the difference in abundance of the binding protein.

Thereafter, proteomic and transcriptomic analyses were used to identify proteins or transcripts more abundant in *dmi3* than in wild-type cell cultures that could correspond to a 100 kDa protein with carbohydrate-binding properties. Based on these criteria, LYR3, LYK9, and LYR4, which were up-regulated at the protein or gene level in *dmi3* compared to wild-type cell cultures, were selected for binding tests.

Nod factor-binding tests performed on LYR3, LYK9, and LYR4, transiently expressed in *N. benthamiana* leaves as fusion proteins with the yellow fluorescent protein (YFP), revealed that only LYR3 exhibited binding. Its binding characteristics were similar to those of NFBS2 in terms of affinity and selectivity toward differentially substituted LCOs (Nod factors and Myc-LCOs) or COs. Therefore, LYR3 was most probably the binding protein in NFBS2. The reason for its higher abundance in the *dmi3* cell culture is unknown but it would be interesting to quantify LYR3 in the roots of wild-type plant and *dmi3* mutant to determine whether the effect observed in cell culture originates from a regulation of *LYR3* by *DMI3* or as a consequence of cell dedifferentiation.

LYR3, similar to other plant LysM-RLKs, has three LysM domains (LysM1, LysM2, LysM3) in the extracellular region, followed by a transmembrane domain and a kinase domain, which, similar to NFP, is predicted to be catalytically inactive. The identification of LYR3 opens the question of the molecular mechanisms involved in the

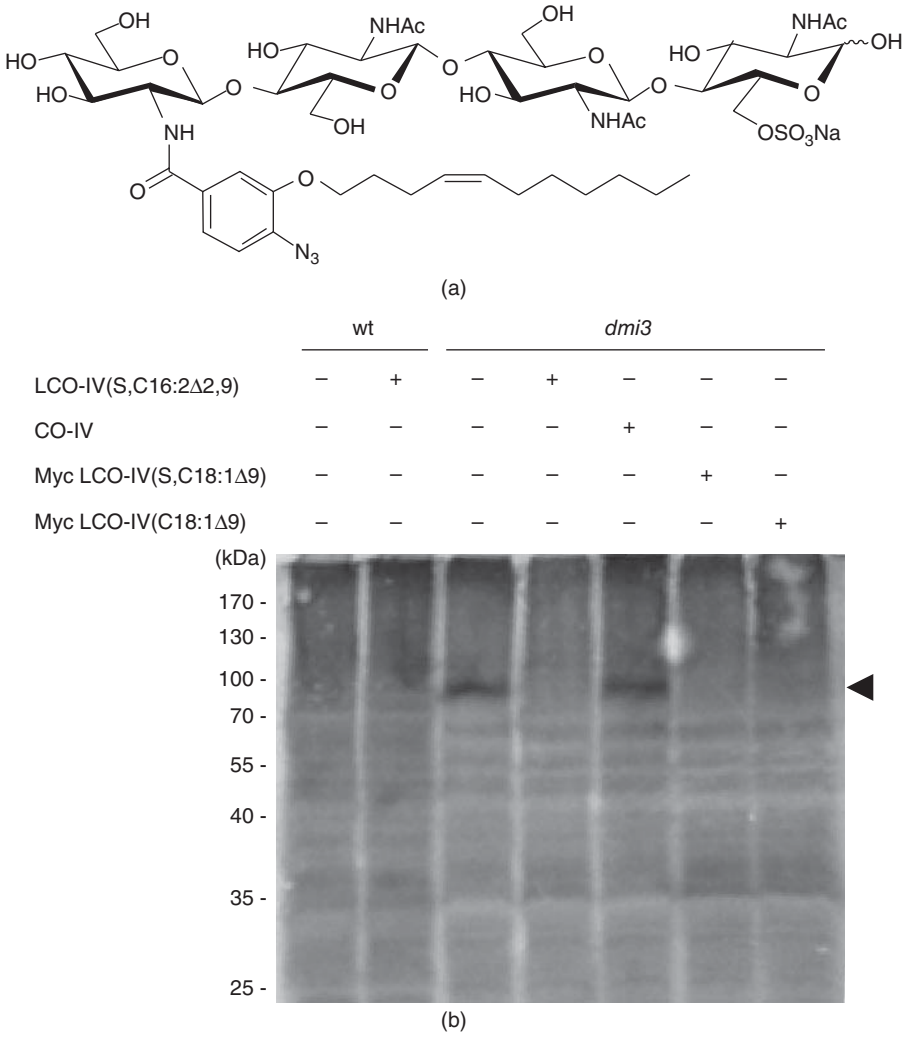

Figure 51.1 Membrane fractions from *M. truncatula* wild-type or *dmi3* cell cultures were incubated with the radioactive aryl-azido photoaffinity probe (a) with or without competing LCOs and COs. After irradiation the proteins were analyzed by SDS-PAGE and autoradiography (b). (Source: Adapted from (Fliegmann et al., 2013) with permission.)

specific recognition of LCOs versus COs. The structure of the extracellular domain of AtCERK1 in complex with chitopentaose, recently solved by X-ray crystallography, showed that the ligand was bound to a groove located on the molecular surface of the second LysM (LysM2) domain, and that the interaction involved three GlcNAc residues via hydrogen bonds with the *N*-acetyl moieties (Liu et al., 2012). Even if the sequence identity between the extracellular domains of LYR3 and AtCERK1 are weak (21.9%), it was sufficient to perform homology modeling and molecular docking with CO and the Nod factor. This approach suggested that the LysM2 domain of LYR3 was structurally close to that of AtCERK1, with the presence of a similar groove able to accommodate CO-IV or sulfated CO-IV. The model predicted that the entire oligosaccharide established contacts with the protein via hydrogen bonds with the *N*-acetyl group of the second and third GlcNAc. The sulfate group was predicted not to interact with the protein surface, which was in accordance with the binding properties of LYR3. This groove is also present in the closest homolog of LYR3 in *A. thaliana*, AtLYK4, involved in chitin perception (Tanaka et al., 2013), suggesting that it is conserved in oligochitin binding proteins. However, it is not present in LYR4, a close homolog of LYR3 in *M. truncatula*, which does not bind the Nod factor. Docking of the Nod factor to the CO binding groove in LYR3 (Fig. 51.2) suggested that the proximal part of the acyl chain may make contact with the LysM2 domain, which was consistent with the photoaffinity labeling of the protein using our probes, in which the photoreactive group was localized in this part of the LCO. However, there was no defined hydrophobic binding pocket for the distal part of the acyl chain that could account for the importance of the length of the fatty acid chain for high-affinity binding of Nod factor to LYR3.

Therefore, this part of the Nod factor could either bind to unknown residues in the LysM2 domain or extend out from the LysM2 domain. The position of the binding groove suggests that it is unlikely to bind to another LysM domain in

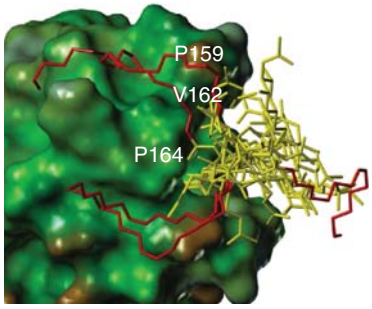

Figure 51.2 Five docking poses for Nod factor interacting with LysM2 of LYR3. Chitin backbone is in yellow and lipid chain is in red. Accessible surface is colored from brown for lipophilic to blue for hydrophilic potential. (Source: Reprinted with permission from (Fliegmann et al., 2013). Copyright 2013 American Chemical Society.)

the same molecule but could perhaps bind to a LysM domain in a homodimer. This mechanism would be similar to the recognition of a chitooctamer by AtCERK1 where the ligand spans across two receptor molecules leading to homodimerization (Liu et al., 2012). Alternatively, we cannot exclude that a different binding site is present in LYR3. Interestingly, it has been reported that the chitin-binding site of the LysM fungal effector protein Ecp6 from *Cladosporium fulvum*, is mediated by intrachain interaction of the LysM1 and LysM3 domains (Sánchez-Vallet et al., 2013).

Our main objective now is to determine the biological function of LYR3. As it does not discriminate Nod factors from Myc-LCOs, it could play a role in the perception of both signals. Another challenge will be to identify the binding proteins in NFBS1 and NFBS3 and to determine whether other LysM-RLKs of *M. truncatula* have the ability to bind Nod factors or Myc-LCOs.

51.5 CONCLUSION

Despite the huge breakthrough made 10 years ago using genetic approaches to identify LysM-RLKs as key players in the establishment of root nodule endosymbioses with Rhizobium, the molecular mechanisms of LCO perception is far from being elucidated. Several lines of evidence indicate that probably multiple component receptors are controlling convergent or independent LCO signaling pathways involved in the establishment of the legume-Rhizobium and arbuscular mycorrhizal symbiosis. Because structurally related ligands (LCO and CO) are recognized by structurally related receptors (LysM proteins) in symbiotic or pathogenic contexts, future work will aim to better understand the role of the extracellular LysM domains and of the transmembrane and kinase domains of the LysM-RLK in the selectivity of ligand binding and receptor activation. To decipher these mechanisms, complementary approaches such as molecular genetics, biochemistry, and cell biology will have to be jointly pursued, which might also integrate the evolutionary history of both plants and their interacting microorganisms.

ACKNOWLEDGMENTS

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Chapter 52

Role of Ectoapyrases in Nodulation

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52.1 APYRASE

Apyrases (Adenosine pyrophosphatase; Enzyme Commission number: 3.6.1.5) are calcium- or magnesium-activated non-energy-coupled nucleotide phosphohydrolases that hydrolyze adenosine triphosphate (ATP) or adenosine diphosphate (ADP) to yield adenosine monophosphate (AMP) and inorganic phosphate. Apyrases have been found in many prokaryotes and eukaryotes (Handa and Guidotti, 1996). As the enzyme hydrolyzes a wide range of nucleoside tri- or di-phosphates, the alternative name, nucleoside triphosphate diphosphohydrolase (NTPDase) has been used more commonly in the field of mammalian research over the last decade (Zimmermann et al., 2000). There are five characteristic apyrase conserved regions (ACRs) described in members of the NTPDase/apyrase family across many prokaryotes and eukaryotes (Handa and Guidotti, 1996; Kirley et al., 2006). ACRs form the active site of apyrases, some of which correspond to the β- and γ-phosphate-binding motifs of the actin/Hsp70/hexokinase superfamily (Hurley, 1996).

In mammals, apyrases function in very diverse biological roles, including blood platelet aggregation, neurotransmission, pathogen–host interaction, cell protein and lipid glycosylation, and oncogenesis (Sarkis and Salto, 1991; Edwards and Gibb, 1993; Marcus and Safier, 1993; Knowles, 2011). Likewise, in plants, apyrases are involved in many aspects of plant physiology, including growth of various tissues (Wolf et al., 2007; Wu et al., 2007; Clark et al., 2010a, 2010b), starch synthesis (Handa and Guidotti, 1996), phosphate metabolism (Thomas et al., 1999), pollen germination and growth (Steinebrunner et al., 2003), regulation of stomatal aperture (Clark et al., 2011), plant–insect interaction in entomogenous galls (Detoni et al., 2012), and plant–microbe interactions with pathogens (Kawahara et al., 2003; Toyoda et al., 2012; Shiraishi, 2013) as well as with symbionts (McAlvin and Stacey, 2005; Govindarajulu et al., 2009; Tanaka et al., 2011a, 2011b; Roberts et al., 2013). These reports suggest that apyrases play essential roles in plant growth, development, and stress response.

Apyrases can be grouped into two classes based on the cellular location of their catalytic domain; that is, endo- and ecto-apyrases. Endoapyrases have an intracellular-located catalytic domain while ectoapyrases have an extracellular catalytic domain (Plesner, 1995). Ectoapyrases are well known to regulate the concentration of nucleotides in the extracellular matrix (Todorov et al., 1997; Marcus et al., 2003). Extracellular nucleotides are important signaling molecules in a number of cellular responses in animals (Khakh and Burnstock, 2009), as well as in plants (Tanaka et al., 2010; Clark and Roux, 2011). These signaling molecules act through specific receptors, the so-called purinoceptors in mammals. However, no obvious homologs of the animal purinoceptors have been found in plants and, to date, the mechanism of extracellular nucleotide recognition by plants has not been defined. A plant extracellular ATP receptor has recently been identified (Choi et al., 2014).

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52.2 ECTOAPYRASE PLAYS AN IMPORTANT ROLE IN NODULATION

A decade ago, phylogenetic analysis identified a legume-specific apyrase family (Cannon et al., 2003) (Fig. 52.1). The authors of this study suggested that these specific apyrases could function in a biological process specific to legumes, such as nodulation. Very recently, the distinctive difference of this legume-specific apyrase group was shown to be due, at least in part, to the amino acid residues comprising the hydrophobic binding pocket for the adenine ring of the substrate (Tanaka et al., 2011a). Some of the legume-specific apyrases were shown to be ectoapyrases, localized to the plasma membrane, and function in the nodulation process (Etzler et al., 1999; Day et al., 2000; Cohn et al., 2001; Govindarajulu et al., 2009; Tanaka et al., 2011a, 2011b). The best characterized legume-specific apyrases are those present in horse gram (*Dolichos biflorus*), soybean (*Glycine max*), and *Lotus japonicus*.

Originally, the ectoapyrase from the legume horse gram was identified based on its lectin-like properties and was subsequently shown to also possess apyrase activity (Etzler et al., 1999). Hence, this protein was named Dolichos biflorus Lectin Nucleotide Phosphohydrolase (DbLNP). As the rhizobial lipo-chitin nodulation signal, the so-called Nod factor (NF) produced by rhizobia, is a short oligosaccharide, it was possible that DbLNP could bind this molecule via its lectin domain. Indeed, DbLNP was found to bind to NFs and other lipo-chitin oligosaccharides derived from various rhizobia (Etzler et al., 1999). The binding increased the enzymatic activity of DbLNP, suggesting a possible signaling role for this enzyme (Etzler et al., 1999). These results were obtained prior to the identification of the LysM-receptor-like kinase proteins, which are now known to be the primary receptors for the NF (Oldroyd, 2013; see Chapter 51). Therefore, at the time of publication, the paper by Etzler et al. (1999) generated quite a bit of interest and speculation that the DbLNP protein could be the NF receptor. Interestingly, the uniform distribution pattern of DbLNP on the surface of young root hairs changed significantly upon rhizobial inoculation to that of tip-localized; the site in which rhizobial infection occurs (Kalsi and Etzler, 2000). This effect on DbLNP location was not seen when non-rhizobial bacteria, including a root pathogen, were added. Moreover, flooding the roots with specific antibodies against the DbLNP protein blocked root hair deformation and nodule formation upon rhizobial inoculation (Etzler et al., 1999; Kalsi and Etzler, 2000). Taken together, these data argued strongly for an early and essential role for DbLNP in the initiation of the symbiotic interaction.

The soybean ectoapyrase GS52, originally identified in the wild soybean *Glycine soja* as a 52 kDa protein, was shown to be induced early not only during rhizobial infection (Day et al., 2000) but also during the later stages of the nodulation process, for example, nodule development stages (Brechenmacher et al., 2008; Govindarajulu et al., 2009). Strong ectopic expression of the *GS52* gene in *L. japonicus* roots resulted in a significant increase in rhizobial infection events in root hairs, again suggesting an early role for this protein in nodulation. The increase in infection events also led to an approximately twofold higher nodule number (McAlvin and Stacey, 2005). Similarly, nodule numbers increased in transgenic soybean and *Medicago truncatula* roots ectopically expressing GS52 (Fig. 52.2).

However, these effects were not seen when a mutant form of GS52, in which amino acid residues critical for its catalytic activity were mutated, was expressed in transgenic roots (Tanaka et al., 2011a). These results indicate that the catalytic activity of the GS52 apyrase, likely acting on extracellular nucleotides, is essential for the stimulation of rhizobial infection and nodulation seen in these transgenic experiments. Furthermore, RNA interference (RNAi)-mediated gene silencing of the *GS52* gene significantly reduced nodule numbers in transgenic soybean roots (Govindarajulu et al., 2009) (Fig. 52.2). These results are consistent with earlier experiments in which flooding roots with a specific anti-GS52 antibody was shown to inhibit rhizobial infection (Day et al., 2000). The reduction in nodule numbers seen in roots in which GS52 was silenced by RNAi could be partially rescued by addition of ADP (Govindarajulu et al., 2009), suggesting that nucleotide signaling, in part mediated by the catalytic activity of GS52, is important for rhizobial infection. Indeed, extracellular ATP is exuded by the root hair tips of *M. truncatula* and this exudation is stimulated by the addition of the rhizobial NF (Kim et al., 2006).

An orthologous ectoapyrase of DbLNP and GS52 was identified from *L. japonicus* by the Etzler laboratory and, following the convention of DbLNP, named LjLNP (Roberts et al., 1999). A very recent study shows that antisense-mediated gene silencing of LjLNP expression blocks NF signaling at the very first steps of plant recognition of this molecule (Roberts et al., 2013). Specifically, the NF-induced perinuclear calcium spiking in the root hair and calcium influx at the root hair tip was abolished in the *LjLNP* antisense transgenic lines. As a result, an early nodulin gene, *NIN,* was not induced and no nodules were formed in the antisense lines, which grew less vigorously than in nodulated control plants in the absence of nitrate or ammonia (Roberts et al., 2013). However, NF- or rhizobium-induced root hair deformation was intact in the transgenic lines, indicating that LjLNP likely acts downstream of the Nod factor receptors NFR1 and NFR5, in which mutations block root hair deformation (Radutoiu et al., 2003). These results also suggest that LjLNP acts upstream or in a parallel position to the other genes in the common symbiotic signaling (SYM) pathway for both nodulation and mycorrhization (SYMRK/DMI2, POLLUX/DMI1 and CASTOR, NUP133, NUP85, NENA)

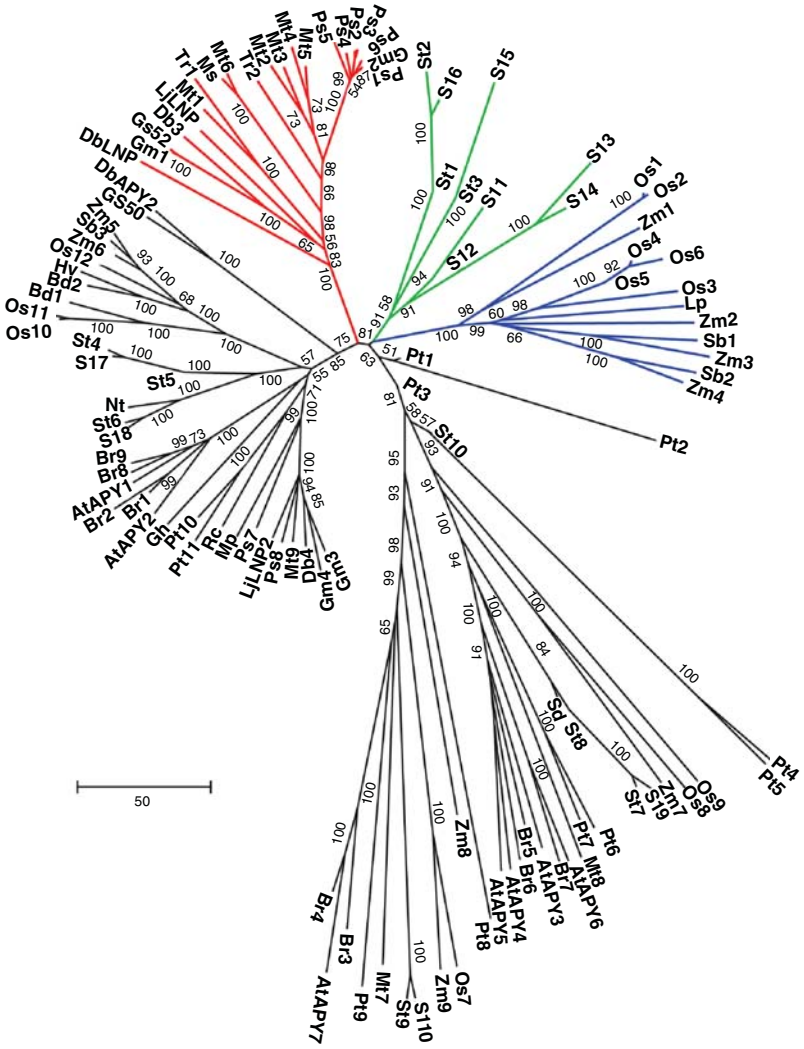

Figure 52.1 Phylogenetic comparison of plant apyrases. The unanchored tree was generated using full length protein sequences of plant apyrases by the neighbor-joining method with 1000 bootstrap replicates. Only bootstrap values >50% are shown in the cladogram at the branching points. Specific apyrase clades, that is, *Fabaceae*-specific clade (red), *Poaceae*-specific clade (blue), and *Solanaceae*-specific clade (green), are indicated in color-highlighted branches. The letters and their associated number correspond to the predicted apyrases with their accession numbers as listed below: **At**: *Arabidopsis thaliana* (AtAPY1, At3g04080; AtAPY2, At5g18280; AtAPY3, At1g14240; AtAPY4, At1g14230; AtAPY5, At1g14250; AtAPY6, At2g02970; AtAPY7, At4g19180), **Bd**: *Brachypodium distachyon* (Bd1, XP_003557997; Bd2, XP_003562437), **Br**: *Brassica rapa* (Br1, Bra002150; Br2, Bra006450; Br3, Bra012554; Br4, Bra013379; Br5, Bra019669; Br6, Bra019670; Br7, Bra024796; Br8, Bra031947; Br9, Bra040151), **Gm**: *Glycine max* (Gm1, XP_003548772; Gm2, BAD13527; Gm3, XP_003548478; Gm4, XP_003553324), **Gs**: *Glycine soja* (GS52, Q9FVC2; GS50, AAG32959), **Gh**: *Gossypium hirsutum* (Gh, ADD09804), **Hv**: *Hordeum vulgare* (Hv, BAJ85095), **Lp**: *Lolium perenne* (Lp, ACB38287), **Lj**: *Lotus japonicus* (LjLNP, AAF00609; LjLNP2, chr1.CM0104.1830.r2.d), **Ms**: *Medicago sativa* (Ms, AAF00611), **Mt**: *Medicago truncatula* (Mt1, Q84UD9; Mt2, Q84UE1; Mt3, Q84UE2; Mt4, MSAF288132; Mt5, Q84UE3; Mt6, XP_003624587; Mt7, XP_003612462; Mt8, XP_003613774; Mt9, AAO23007), **Mp**: *Mimosa pudica* (Mp, BAK78981), **Nt**: *Nicotiana tabacum* (Nt, ABK51386), **Os**: *Oryza sativa* (Os1, NP_001067813; Os2, EAY96392; Os3, EEC67580; Os4, ABA91303; Os5, ABA95699; Os6, NP_001066032; Os7, NP_001064408; Os8, NP_001061886; Os9, AAL82534; Os10, EEC75177; Os11, EAZ26787; Os12, EEC82701), **Ps**: *Pisum sativum* (Ps1, AB038669; Ps2, BAB87182; Ps3, BAB87198; Ps4, BAB87197; Ps5, BAD13517; Ps6, BAD13519; Ps7, AAG22044; Ps8, BAB85978), **Pt**: *Populus trichocarpa* (Pt1, Potri.T151300; Pt2, Potri.019G031200; Pt3, Potri.013G053700; Pt4, Potri.004G195400; Pt5, Potri.009G157500; Pt6, Potri.008G086400; Pt7, Potri.010G169100; Pt8, Potri.015G079900; Pt9, Potri.004G236900; Pt10, Potri.019G031000; Pt11, Potri.013G053500), **Rc**: *Ricinus communis* (Rc, XP_002525470), **Sd**: *Solanum demissum* (Sd, AAT66769), **Sl**: *Solanum lycopersicum* (Sl1, Solyc02g032550; Sl2, Solyc02g032560; Sl3, Solyc12g098540; Sl4, Solyc12g098550; Sl5, Solyc09g092400; Sl6, Solyc02g081980; Sl7, Solyc08g016010; Sl8, Solyc12g096560; Sl9, Solyc05g007670; Sl10, Solyc05g052490), **St**: *Solanum tuberosum* (St1, PGSC0003DMT400018918; St2, PGSC0003DMT400018919; St3, PGSC0003DMT400053021; St4, PGSC0003DMT400019809; St5, PGSC0003DMT400019810; St6, PGSC0003DMT400075516; St7, PGSC0003DMT400047384; St8, PGSC0003DMT400047383; St9, PGSC0003DMT400069878; St10, PGSC0003DMT400093747), **Sb**: *Sorghum bicolour* (Sb1, XP_002442736; Sb2, XP_002448918; Sb3, XP_002461227), **Tr**: *Trifolium repens* (Tr1, ACB38285; Tr2, ACB38286), **Db**: *Dolichos biflorus* (DbLNP, AF139807; DbAPY2, AAF00610; Db3, Q5NT85; Db4, BAD80837), **Zm**: *Zea mays* (Zm1, GRMZM2G349839; Zm2, GRMZM2G085711; Zm3, GRMZM2G431714; Zm4, GRMZM2G006762; Zm5, GRMZM2G131026; Zm6, GRMZM2G003331; Zm7, GRMZM2G152457; Zm8, GRMZM2G106618; Zm9, GRMZM2G140587).

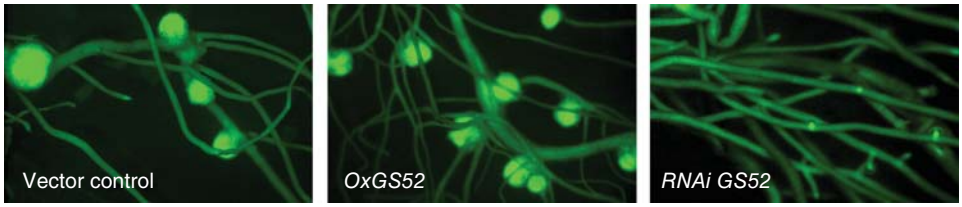

Figure 52.2 Nodule formation on transgenic soybean roots. Each figure shows transgenic soybean roots 4 weeks after inoculation with the soybean symbiont, *Bradyrhizobium japonicum*. The panels represent (a–c) transgenic roots carrying the empty vector (vector control), ectopically expressing *GS52* from a strong, constitutive promoter (*OxGS52*), or expressing an RNAi construct silencing *GS52* expression (*RNAi GS52*). The transgenic roots could be identified due to the constitutive expression of the green fluorescent protein, GFP. Note that, in comparison with the transgenic control roots the transgenic roots ectopically expressing *GS52* produced more nodules, whereas the transgenic roots in which *GS52* expression was silenced showed a severe reduction in nodulation. For more detail, see Govindarajulu et al. (2009) and Tanaka et al. (2011a).

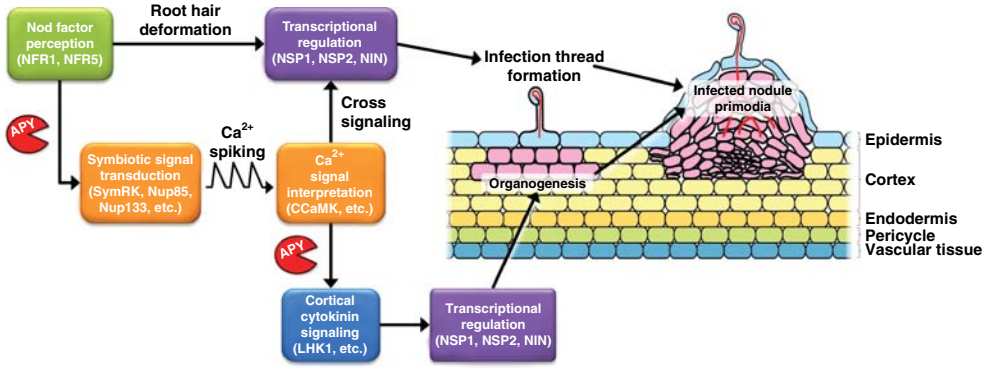

Figure 52.3 Model of ectoapyrase function in the symbiotic signaling pathway during the nodulation process. Regulatory pathways associated with rhizobium infection (root hair deformation, infection thread formation) and nodule organogenesis (cortical cell division) lead to nodule primordium formation as exemplified for determinate nodule formation on *L. japonicus* roots in the figure. Ectoapyrase (APY) is proposed to act upstream of the common SYM pathway (shown in orange box; e.g., SymRK) but downstream of the Nod factor receptors (NFR1 and NFR5). We also propose that ectoapyrase (APY) might be involved in regulating hormonal levels (i.e., cytokinins) for nodule organogenesis, although this hypothesis has to be confirmed by experimental evidence. Research studies performed on the non-legume Arabidopsis suggest that ectoapyrases are involved in signaling/regulation of plant hormones.

(Parniske, 2008; Singh and Parniske, 2012) (Fig. 52.3). Mutations in these genes also block NF-induced calcium spiking but still show NF-induced root hair calcium influx and root hair deformation. A role in the common SYM pathway, which is shared between the rhizobial and mycorrhizal symbioses, is also strongly supported by the demonstration that silencing of LjLNP also blocked infection of arbuscular mycorrhizal (AM) fungi (Roberts et al., 2013).

Collectively, the above-mentioned published data clearly demonstrate an important role for legume-specific ectoapyrases in nodulation and the mycorrhizal symbiosis. It remains to be seen whether ectoapyrases also function in mycorrhizal infection in those non-legume plants, which lack this legume-specific ectoapyrase family, and yet are infected by AM fungi.

52.3 POSSIBLE FUNCTIONS OF ECTOAPYRASE IN NODULATION SIGNALING

As mentioned above, before the identification of the NF receptors NFR1/LYK3 and NFR5/NFP (Nod-factor perception), ectoapyrases were viable candidates for the long-sought NF receptor (Geurts and Bisseling, 2002). Legume ectoapyrases were found to directly bind NFs and plants showed nodulation-deficient phenotypes when the enzyme function was blocked by specific antibodies (Etzler et al., 1999; Day et al., 2000). NF binding by DbLNP increased apyrase activity and, therefore, conceivably, this effect could be essential for subsequent NF-induced cellular signaling. However, other than hydrolysis of extracellular

nucleotides, there remains no solid biochemical connection between ectoapyrase activity and nodulation signaling.

We have previously postulated that ectoapyrase is important in nodulation due to the ability to control the level of extracellular nucleotides at the root hair surface. The addition of NFs, as well as known pathogen elicitors (including chitin), increases extracellular ATP release (Kim et al., 2006; Wu et al., 2008; Tanaka et al., 2011a). Treatment of plants with extracellular ATP was also shown to change their susceptibility to pathogen infection (Song et al., 2006). One hypothesis is that the ectoapyrase enzyme decreases extracellular ATP levels induced upon symbiont infection and thereby prevents the activation of plant defense pathways that could limit symbiont invasion (Fig. 52.4).

Studies in the non-legume *Arabidopsis* have shown that ATP (but not ADP) does induce the production of reactive oxygen species (ROS) in root hairs (Clark et al., 2010b) and epidermal cells (Demidchik et al., 2011). ROS are well-known signals important in plant defense response against pathogens. However, ROS are also important for a variety of other plant processes, including cell wall growth (Brewin, 2004; Jamet et al., 2007; Kopcińska, 2009), which could be important during root hair curling and in the formation of the infection thread, by which the rhizobia gain entry into the interior of the root.

NF control of ectoapyrase function may be complex as this molecule has been shown to induce ectoapyrase gene expression (Day et al., 2000), change the distribution of the ectoapyrase protein on the root hair surface (Kalsi and Etzler, 2000), as well as increase DbLNP enzymatic activity (Etzler et al., 1999). It should be noted that addition of NF to the soybean GS52 apyrase did not result in increased enzymatic activity so it remains to be seen if this is a general property of the legume-specific ectoapyrase family (Tanaka et al., 2011b).

The reduction in nodulation seen upon RNAi silencing of the GS52 ectoapyrase in transgenic soybean roots could be partially restored upon the addition of exogenous ADP. These data suggest that it may be the catalytic products of apyrase action that are critical for the nodulation process, perhaps acting as an extracellularly generated signal (Fig. 52.4). Indeed, as already mentioned, the catalytic domain of the GS52 apyrase was shown to be essential for the ability required to enhance nodulation when the protein was ectopically expressed in transgenic soybean roots (Tanaka et al., 2011a). Consistent with such a notion, the addition of ADP to root hairs was shown to change plasma membrane conductance (Lew and Dearnaley, 2000). ADP treatment was also shown to induce calcium influx in the root epidermal cells (Demidchik et al., 2011). In this case, ADP was more effective than extracellular ATP, for example, half-maximal depolarization is at 0.4 mM for ATP and at 10 μM for ADP (Lew and Dearnaley, 2000). Similar results would be seen during the nodulation process (Fig. 52.4). NF-triggered release of nucleotides may be causal for some of the early, nodulation-related cellular responses, with ectoapyrases acting to modulate the levels of extracellular nucleotides to maximize their effects. It is likely that any test of this hypothesis will require a more detailed understanding of the basic mechanisms of extracellular nucleotide recognition by plants and the subsequent downstream signaling events. For example, the identification of extracellular nucleotide receptors in plants would be a major advance.

It is also possible that ectoapyrases are involved in organogenesis (Fig. 52.3) and in maintaining functional nodules as *GS52* gene expression is continuously upregulated during the entire nodulation process (Brechenmacher et al., 2008; Govindarajulu et al., 2009). Nodule organogenesis is also regulated by plant hormones; for example, cytokinin signaling is especially important (Oldroyd and Downie, 2008). Moreover, asymmetric distribution of auxin has been observed at the onset of organogenesis (Boot et al., 1999; Takanashi et al., 2011). It is interesting to speculate that ectoapyrase might indirectly (mediated through controlling extracellular ATP level) regulate hormonal actions during the nodule organogenesis process. Perhaps consistent with this notion, the transgenic expression of the pea ectoapyrase in Arabidopsis was found to increase the resistance to the toxic levels of cytokinin (Thomas et al., 2000), implying that the hormonal action could be influenced by the activity of this enzyme. In other respects, apyrase was implicated as a positive regulator of polar auxin transport, thereby changing the asymmetric distribution of auxin in *Arabidopsis* roots (Liu et al., 2012). However, there is no direct evidence in legume plants in terms of the relationships between ectoapyrase enzymatic activity, hormonal levels, and nodule organogenesis.

A final role may be that ectoapyrases can supply phosphorus to the plant upon hydrolysis of ATP or other nucleotides. This assumption is supported by numerous reports showing that phosphorus is required for cortical cell division and organogenesis during the nodulation process (de Mooy and Pesek, 1966; Israel, 1987; Tang et al., 2001; Gentili et al., 2006; Miao et al., 2007).

52.4 FUTURE PERSPECTIVES

The recent paper by Roberts et al. (2013) demonstrated that ectoapyrases are essential for both rhizobial and mycorrhizal symbioses. Given that mycorrhizal associations have been documented as early as circa 400 million years ago (Parniske, 2008), it is likely that ectoapyrases initially evolved to play a role in AM signaling, and were then secondarily recruited, along with other members of the common SYM pathway, to play a role in nodulation. Indeed, apyrase genes in AM host plants (*Fabaceae* (legume), *Poaceae* (monocot),

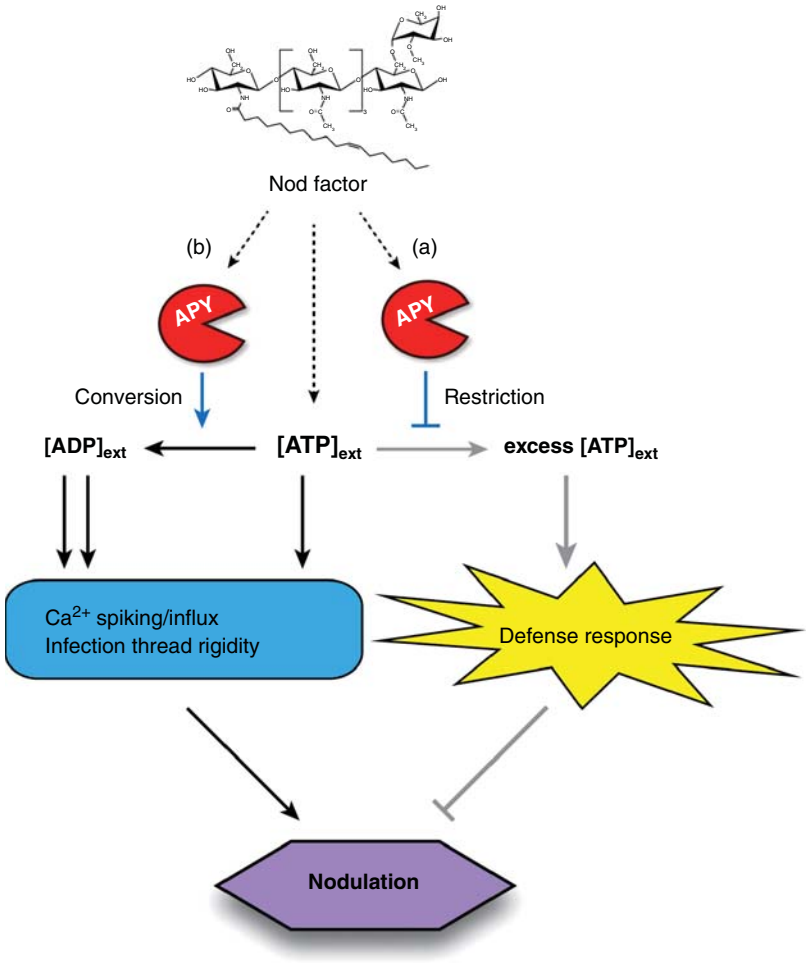

Figure 52.4 Hypothetical role for ectoapyrases in maintaining an optimal extracellular nucleotide concentration to allow rhizobial infection. Ectoapyrase either reduces the extracellular ATP level or increases the level of ATP degradation products (e.g., ADP and others), thereby affecting root symbiotic signaling. (a) Rhizobial infection or Nod factor addition increases the extracellular ATP concentration ($[ATP]_{ext}$) at the root hair surface (Tanaka et al., 2011a). Concurrently, the Nod factor maximizes the ectoapyrase (APY) function by increasing its gene expression (Day et al., 2000), by changing its localization (Kalsi and Etzler, 2000), or by directly affecting its enzymatic activity (Etzler et al., 1999) in the root hair. The ectoapyrase then restricts $[ATP]_{ext}$ at an optimal level to prevent the induction of plant defense responses. For example, the addition of ATP induces excess amount of ROS production in the root hair surface (Kim et al., 2006; Cardenas et al., 2008). (b) Alternatively, the activated ectoapyrase generates degradation products of ATP, for example, ADP and others, that might be important for symbiotic signaling. For example, extracellular ADP was shown to change plasma membrane conductance in root hairs (Lew and Dearnaley, 2000) and induce calcium influx in root epidermal cells (Demidchik et al., 2011). ADP addition could be more effective than ATP in stimulating nodulation.

and *Solanaceae* species) but not non-AM host (e.g., *Brassicaceae* species), are classified in specific clades (Fig. 52.1). This phylogenetic classification fits the symbiotic toolkit model proposed by Delaux et al. (2013); that is, symbiotic ectoapyrases can be molecular components of the symbiotic toolkit, a core set of symbiotic genes conserved in all AM host plants (Delaux et al., 2013). This raises the interesting future possibility that identification of all members of this toolkit could eventually provide the means to transfer mycorrhizal and rhizobial symbiotic capability to those plant species that currently lack these abilities (see Chapter 108). This is clearly more critical for nodulation, given that a large number of plants are mycorrhizal. Very interestingly, transgenic expression of the soybean ectoapyrase GS52 enhanced colonization of rhizobia surrounding the tip area of root hairs in rice plants (Sreevidya et al., 2005). Because many monocots are infected by AM fungi, they must already contain many components of the common SYM pathway (Venkateshwaran et al., 2013). Introducing the legume-specific ectoapyrases into monocot plants may be an essential step toward the ultimate goal of engineering nitrogen-fixing non-legume crop plants. The idea of creating nitrogen-fixing, non-legume crop plants has been a "Holy Grail" of the field of biological nitrogen fixation (see Chapter 108). It is of wider general interest due to the importance of biological nitrogen fixation and its tremendous agronomic potential if we could achieve fixation in non-legumes. Understanding ectoapyrase function, acting on extracellular nucleotides, during the symbiosis process would also aid in the engineering of more efficient associations between crops and microbes for sustainable agriculture in the future.

As described above, recent research findings have provided significant evidence regarding the role of ectoapyrase in plant–symbiont interactions, where the activity of ectoapyrase, controlling extracellular nucleotide levels, is essential for maximizing symbiotic signaling. However, there are still many open questions about the detailed mechanism, that is, how extracellular nucleotide excretion and possible signaling is involved in rhizobium–symbiotic signaling. Although the model we proposed (Figs. 52.3 and 52.4) is consistent with current evidence, key features still lack strong experimental support, for example, what is the extracellular ATP receptor in plants, which will be addressed by ongoing research efforts in many laboratories.

ACKNOWLEDGMENTS

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Chapter 53

Role of *Rhizobium* Cellulase CelC2 in Host Root Colonization and Infection

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53.1 INTRODUCTION

Among the many factors involved in the development of an effective symbiosis between rhizobia and their host plants, those associated with adherence, colonization, and infection involving entry into the plant have not been fully elucidated at the molecular level (Robledo et al., 2012).

Root attachment and colonization in rhizobia follows the two-step system described in bacteria (Dazzo et al., 1984). During early reversible specific binding events, several bacterial proteins have been proposed to participate, such as bacterial ricahdesin and flagella, and plant lectins (Dazzo et al., 1984, Ausmees et al., 2001; Fujishige et al., 2006; De Hoff et al., 2009), while polysaccharides are the main components involved in the later irreversible attachment stages. Some of these cell surface components include exopolysaccharides, lipopolysaccharides, cyclic β-1,2-glucans (Davey and O'Toole, 2000), and especially cellulose fibrils that mediate aggregation to root hairs and firmly anchor the bacteria to the root surface (Dazzo et al., 1984; Smit et al., 1992; Mateos et al., 1995; Robledo et al., 2012). Attachment to a surface is the initial step in biofilm formation, followed by cooperative colonization behaviors leading to the establishment of microcolonies by clonal propagation and maturation, and formation of three dimensional structures that are embedded in and covered by exopolymers (Costerton et al., 1987; Davey and O'Toole, 2000; Webb et al., 2003; Fujishige et al., 2006). Studies have revealed that *Rhizobium* (Napoli et al., 1975) as well as species of the *Enterobacteriaceae* family, such as *Citrobacter* spp., *Enterobacter* spp., and *Klebsiella* spp., produce cellulose as a crucial component of the bacterial flocs and extracellular matrix (reviewed in Lasa 2006; Robledo et al., 2012).

Infection is a term considered by most to mean the entrance, growth, and multiplication of microorganisms within a host, resulting in the establishment of a disease

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process. Others define infection as the ability to breach the host barrier and colonize internal host tissues, regardless of whether it leads to detectable disease symptoms or not. This is the case of rhizobia. These beneficial bacteria infect host plants and promote their growth instead of producing disease. For this to occur, there must be full coordination between the macro and microsymbionts (Mateos et al., 2011). Rhizobia invade their plant hosts through colonization of intercellular epidermal spaces, crack entry at emerging lateral roots or penetration into deformed tips of host root hairs to produce tubular walled structures called infection threads (Dazzo and Wopereis, 2000; Goormachtig et al., 2004).

A key event of the primary infection process required for the development of the *Rhizobium*-legume root-nodule symbiosis is the passage of the bacteria across the root hair wall. This process of host wall degradation must be highly localized and delicately balanced in order for the bacterial symbiont to fully penetrate and trigger infection thread formation successfully without killing the root hair and subsequent abortion of the infection process (Peix et al., 2010). Several hypotheses have been proposed to explain how this event occurs. Nutman et al. (1973) proposed that rhizobia redirect growth of the root hair wall from the tip to the localized site of infection, causing invagination of an intact root hair wall rather than bacterial penetration of it. Ljunggren and Fähraeus (1961) proposed that homologous *Rhizobium* strains specifically induce the host plant to produce polygalacturonases, which soften the root hair wall at the site of infection, thus allowing the bacteria to penetrate between microfibrils to the cell membrane and initiate an infection thread. The third model (Hubbell, 1981) proposed that wall-degrading enzymes produce a localized degradation that completely traverses the root hair wall, creating a continuous portal of entry allowing direct penetration by the bacteria. The strongest evidence for involvement of wall hydrolysis in the *Rhizobium leguminosarum* bv trifolii-white clover infection process was obtained by Callaham and Torrey (1981), who reported a localized degradation of the root hair wall coincident with the deposition of a new wall layer, above the site of degradation, which is continuous with the root hair wall. The main point of divergence would be the possibility that the wall-degrading enzymes involved in the process are associated with the bacteria or locally induced in the plant by components of the bacteria (Peix et al., 2010). Plant cell wall-degrading enzymes are predicted to participate in two steps of this infection process: during primary infection of the host root hairs leading to infection thread formation (Inf) and later during bacterial release (Bar) from walled infection threads within host nodule cells (Robledo et al., 2008, 2011a).

Extending the role of hydrolytic enzymes in the active penetration of plant cell walls by some pathogenic microorganisms, McCoy (1932) was the first to investigate the possible involvement of hydrolytic enzymes in the infection of legumes by rhizobia. There was no evidence of these enzymes from rhizobia, though sensitive procedures to detect minute amounts of cell wall-degrading enzymes were not yet available. Later, several studies detected pectinolytic (Hubbell et al., 1978), cellulolytic (Morales et al., 1984), and hemicellulolytic (Martínez-Molina et al., 1979) enzyme activities from pure cultures of rhizobia. In general, the activities of these rhizobial enzymes are very low and at the limit of sensitivity of conventional reducing sugar assays. Using improved, reliable assays of increased sensitivity (Mateos et al., 1992), it was established that cellulases are produced by all type strains of the official species of rhizobia (Jiménez-Zurdo et al., 1996; Robledo et al., 2008). In contrast, polygalacturonase activity is less commonly found in rhizobia (Jiménez-Zurdo et al., 1996). Progression of infection threads across adjacent root cells would require the sequential action of plant enzymes degrading other components of the cell-wall induced upon rhizobia inoculation, such as by plant polygalacturonases in *Medicago sativa* and *Medicago truncatula* (Muñoz et al., 1998; Rodríguez-Llorente et al., 2003) and pectate lyases in *Lotus japonicus* (Xie et al., 2012).

Two key findings in earlier studies were the *in vivo* cell-bound location rather than extracellular secretion of these enzymes, and the substrate requirement of being amorphous rather than non-crystalline (Mateos et al., 1992, 2001). These two characteristics of the rhizobial enzyme have profound implications on their predicted role in primary host infection.

53.2 METHODS

All methods of DNA manipulation, construction of strain derivatives, detection, purification and biological activities of enzymes and substrates, plant microbiology, and determination of biofilm formation are described in (Robledo et al., 2008, 2011a, 2012).

53.3 RESULTS AND DISCUSSION

53.3.1 Cellulases in Rhizobia

The cellulase genes and the cellulolytic enzymes they encode are widespread and commonly expressed among bacteria establishing the root nodule symbiosis (Robledo et al., 2008, 2011b). Among them, the *celC* gene, located near putative cellulose synthase genes in a region of the chromosome (*celABC*), is involved in bacterial cellulose biosynthesis (Robledo et al., 2012) and encodes a cellulase (CelC2) essential for the symbiotic infection of legume host roots in the genus *Rhizobium* fulfilling a very significant

role in infection processes required for the development of the nitrogen-fixation endosymbiosis (Robledo et al., 2008, 2011a). All species in the genus *Rhizobium* analyzed up to date carry a *celC* gene in their chromosome (Robledo et al., 2011b). Furthermore, the phylogeny of the *celC* gene is completely congruent with those based on the chromosomal genes, but not with those based on plasmid-borne genes, in species of the genus *Rhizobium*, and thus *celC* represents a new phylogenetic marker useful for taxonomic studies in this genus (Robledo et al., 2011b).

53.3.2 Role of *Rhizobium* Cellulase CelC2 in Root Colonization

Among the molecules involved in biofilm development are different proteins and some exopolysaccharides, such as cellulose, which is a major component of the biofilm matrix of several bacterial species (Romling, 2002). It is not known how well conserved cellulose production is among all currently known bacterial genera or species that can establish symbiotic nitrogen-fixing root nodules with legumes. Currently, the known diversity of these bacteria mostly resides within the order *Rhizobiales*, which includes about 83 species distributed in different families and genera. We examined most of the official type strains of each of these taxa for cellulose production by Congo red staining, and all were found to be positive to varying degrees (Robledo et al., 2012). We previously used direct *in situ* enzyme cytochemistry at subcellular resolution to detect cellulose microfibrils associated with wild-type *Rhizobium leguminosarum* bv. trifolii ANU843 cells anchored on the roots of its host plant, white clover (Mateos et al., 1995). Timing of their production coincides with the transition from loose to firm adhesion of the bacteria to clover root hairs *in planta* (Dazzo et al., 1984).

By testing cellulose production and biofilm formation by rhizobia on different abiotic *substrata* and examining their attachment to the root epidermis of the host plant clover, we qualitatively and quantitatively compared the biofilm formation ability of the wild type strain ANU843 and its derivatives ANU843ΔC2 (CelC2 knockout) and ANU843C2+ (CelC2 overproducing), illustrated in Figure 53.1.

Aggregation of bacteria, usually mediated by cellulose microfibrils, and dispersion of cell aggregates by exogenously added cellulase are highly suggestive that an excess of extracellular cellulose was being produced (Deinema and Zevenhuizen, 1971; Napoli et al., 1975). The ANU843ΔC2 mutant flocculated heavily into large cell aggregates in yeast mannitol broth (YMB) liquid culture, and these flocs were completely dispersed after treatment with commercial cellulase (Sigma) for 2 h (Robledo et al., 2012). In contrast, flocculation was visually undetectable in the CelC2 overproducing derivative strain ANU843C2+ (Robledo et al., 2012).

Intensity of Congo red staining of ANU843ΔC2 and ANU843C2+ strains was greatly increased or reduced, respectively, as compared to the wild type (Fig. 53.1a). In addition, quantitative image analysis of fluorescence micrographs of cultures stained with the fluorochrome Calcofluor, which binds to β-linked glucans such as cellulose, indicated that the extracellular microfibrils associated with cells of the ANU843ΔC2 mutant were Calcofluor-bright and significantly longer than the average length associated with wild-type cells (Robledo et al., 2012), and that they were not detected in association with the ANU843C2+ derivative strain (Fig. 53.1b).

Thus, the increase in flocculation, enzymatic treatment, Congo red staining, and estimated microfibril length observed in the ANU843ΔC2 mutant are all directly correlated with the overproduction of external cellulose microfibrils. By contrast, the *celC* over-expression derivative strain lost the ability to make extracellular cellulose microfibrils (Robledo et al., 2012). These results suggest that *R. leguminosarum* bv. trifolii cellulase CelC2 is involved in the elongation of cellulose extracellular microfibrils (Robledo et al., 2012).

Biofilm rings of ANU843ΔC2 at the glass–liquid–air interface were thicker, more compact, and more easily

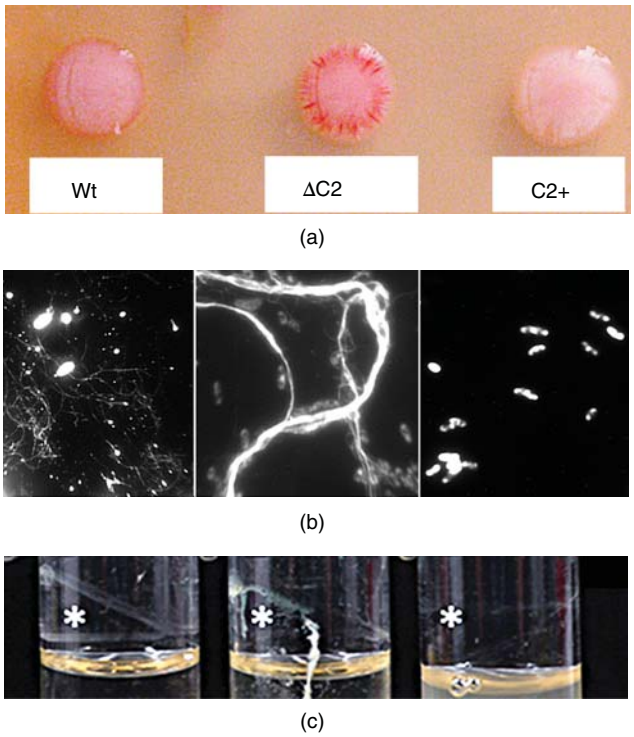

Figure 53.1 Role of *Rhizobium* cellulase CelC2 in cellulose biosynthesis and biofilm formation. (a) Colony uptake of Congo red, (b) calcofluor staining of cells and extracellular microfibrils, and (c) ring formation at the glass–air–liquid interface by wild-type strain of *Rhizobium leguminosarum* bv. trifolii ANU843 (wt) and its derivatives ANU843ΔC2 (ΔC2) and ANU843C2+ (C2+).

dislodged from the glass surface compared to the wild type strain (Fig. 53.1c). In ANU843C2+, the rings were much thinner, suggesting a role for CelC2 in biofilm formation (Robledo et al., 2012).

We also examined biofilm development on a sand substratum representing a more natural environment for these bacteria. Polyvinyl chloride (PVC) tabs were also used to examine the three-dimensional structure of biofilms formed at the edges of inert surfaces. Wild type ANU843 developed microcolonies, which coalesced into a characteristic three-dimensional biofilm (Robledo et al., 2012). In contrast, ANU843ΔC2 established unstable aggregates on surfaces that were easily removed (Robledo et al., 2012). On the other hand, ANU843C2+ bacteria only produced a two-dimensional monolayer biofilm, with just a few bacteria attached to one another (Robledo et al., 2012).

These results confirm the role of external cellulose microfibrils in biofilm attachment and architecture. They further suggest that *R. leguminosarum* bv. trifolii does not develop biofilm normally if production of cellulose microfibrils is altered by elevated or diminished levels of CelC2 endoglucanase (Robledo et al., 2012).

To extend these results found in the abiotic surface tests, we also examined attachment and biofilm formation of *Rhizobium leguminosarum* bv. trifolii and its *celC* derivative strains on roots and root hair tips of the host plant *Trifolium repens* grown in a N-free Fähraeus medium. All strains colonized the root, especially root hair zone II, and remained attached to the root surface even after thorough washing (Robledo et al., 2012). Even strain ANU843C2+ impaired in external cellulose microfibrils biosynthesis attached to plant roots, further confirming that bacterial cellulose does not act alone in plant root attachment (Robledo et al., 2012).

Most root hairs in the growing root hair zone were covered with wild type *R. leguminosarum* bv. trifolii ANU843 cells growing in a three-dimensional biofilm (Robledo et al., 2012) whereas ANU843ΔC2 formed large aggregates that covered the root in an irregular manner (Robledo et al., 2012). In contrast, very few individual cells of the ANU843C2+ derivative strain (cellulose overproducing) were attached to root hairs (Robledo et al., 2012).

The differences in the pattern of root and root hair attachment by the cellulose mutants imply that the cellulose fibrils play a significant role in the stability of the three-dimensional root hair "cap" biofilm (Robledo et al., 2012).

53.3.3 Role of *Rhizobium* Cellulase CelC2 in Root Infection

We have purified the cell-bound cellulase isozymes CelC2 from *R. leguminosarum* bv. trifolii to homogeneity, and analyzed its symbiotic function by a combination of biochemical, reverse genetics and plant microscopy approaches.

Rhizobial CelC2 is a 1,4-ß-D-endoglucanase (EC 3.2.1.4) with high substrate specificity for non-crystalline (amorphous) cellulose. It has an approximate molecular mass of 33.2 kDa, an optimal pH of 5.0, a maximal rate of carboxymethyl-cellulose (CMC) hydrolysis at 40 °C, and an apparent Km of 84.4 mg/ml for CMC as the substrate (Robledo et al., 2008). These biochemical characteristics of CelC2 cellulase restrict its symbiotically relevant activity during primary host infection. The cell-bound location, the high Km value, and the relatively low activity of CelC2 cellulase are all characteristics that would restrain its degradative action on roots, thereby minimizing indiscriminate host cell lyses and death (Mateos et al., 2011). The substrate specificity of CelC2 cellulase for non-crystalline cellulose significantly restricts its *in vivo* site of wall-penetrating erosive action to the highly localized root hair infection sites lacking crystalline wall architecture (Mateos et al., 2001; Robledo et al., 2011a). The pH 5 optimum for CelC2 cellulase is consistent with the slightly acidic pH at the external surface of white clover root hairs. Finally, the specificity in the host plant exhibited by the Hot (Hole on the tip) biological activity of CelC2 cellulase, which includes the compatible white clover legume but excludes the heterologous, non-host legume alfalfa, is consistent with the host specificity of infection thread formation in legume root hairs (Robledo et al., 2008).

Purified CelC2 can completely erode the highly localized non-crystalline tip of the host root hair, forming a complete hole whose geometry and location match the portal of entry point in primary host infection into white clover (Mateos et al., 2001; Robledo et al., 2008). CelC2 knockout mutants are unable to breach the host wall at the root-hair tip or form infection threads within the host root hair and effective nodules (Robledo et al., 2008). Transfer of the cloned wild-type gene into the CelC2 knockout mutant restored these symbiotic phenotypes (Robledo et al., 2008).

Plants grew well in nitrogen-free medium when inoculated with the wild type strain ANU843, indicative of an effective nitrogen-fixing symbiosis. Those plants benefited by symbiotic nitrogen-fixation producing greener leaves and shoots that were significantly longer than plants inoculated with the mutant ANU843ΔC2 or the uninoculated axenic control plants (Robledo et al., 2008). The latter two groups of plants were stunted in development and became chlorotic once the fixed nitrogen from their cotyledons was exhausted, indicative of nitrogen starvation stress without the benefit of nitrogen fixation under these N-free growth conditions (Robledo et al., 2008). Complementation of the mutant strain ANU843ΔC2 with a plasmid expressing the *celC* gene restored the ability to produce the CelC2 cellulase isoenzyme, infect root hairs, and induce effective nitrogen-fixing nodules on white clover roots (Robledo et al., 2008).

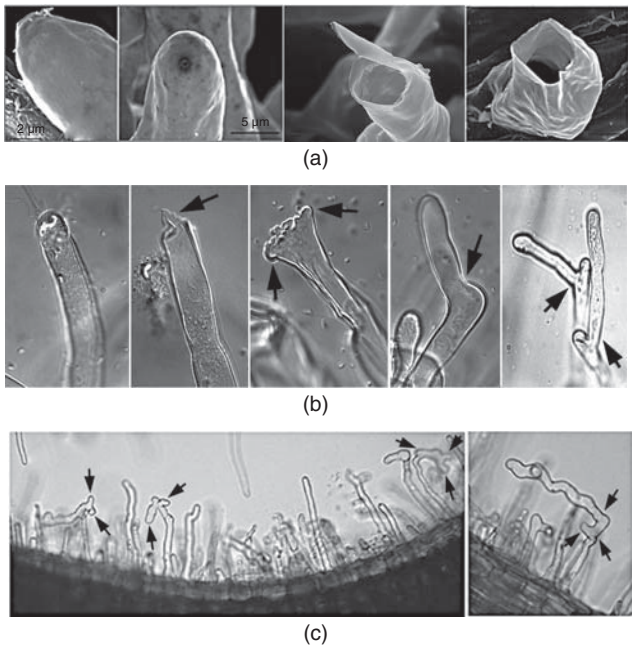

Figure 53.2 Effect of *Rhizobium* cellulase CelC2 overproduction on primary infection. (a) Scanning electron microscope (SEM) images showing the Hot (Hole on the tip) phenotype on root hairs of white clover inoculated with ANU843C2+. (b) The phase-contrast and Nomarski interference contrast micrographs show the deposition of new cell wall material before *transmuro* erosion of the original root hair tip followed by redirection of new tip growth (arrows) resulting in the development of a branch in the root hair that is again susceptible to hydrolysis at its new growing tip. (c) Clover root hairs showing multiple redirections (arrows) after inoculation with ANU843C2+.

These results provide compelling evidence that this enzyme fulfills a very significant role in the primary infection process required for the development of the canonical nitrogen-fixing *R. leguminosarum* bv. trifolii-white clover symbiosis (Robledo et al., 2008).

We have used a gain-of-function approach to further explore the impact of the CelC2 activity on the infection process during the development of the white clover-rhizobia symbiosis (Robledo et al., 2011a). Root hair deformation and infection thread formation assays were performed on white clover plants inoculated with cells of the wild-type strain of *R. leguminosarum* bv. trifolii ANU843, ANU843EV (Empty Vector) control or the recombinant strain that over produces CelC2 (Robledo et al., 2011a). ANU843C2+ cells induced various alterations at the root hair tip that included: (i) canonical primary infection stages as occurs in wild type or ANU843EV with deformations, markedly curled shepherd's crooks and infection thread formation (Robledo et al., 2011a) and (ii) hot phenotype as occurs in response to incubation with purified CelC2 enzyme (Robledo et al., 2011a) where the wall at the tip of some growing root hairs is degraded to produce a hole at this location (Fig. 53.2a) resulting in the extrusion of the root hair protoplast/cytoplasm. Although root hairs without tips can be found at very low frequency in uninoculated plants or after inoculation with the wild type strain, this feature is more frequent after ANU843C2+ inoculation (Robledo et al., 2011a); (iii) sometimes, new cell wall material was deposited before *transmuro* erosion of the original root hair apex, followed by redirection of new tip growth resulting in the development of a branch in the root hair (Fig. 53.2b) that is again susceptible to hydrolysis at its new growing tip (Fig. 53.2c). These branched redirections of root hair growth were also frequently observed on axenic clover roots after 12 h of incubation with purified cellulase CelC2 (Robledo et al., 2011a). These features show that the CelC2 cellulase-overproducing strain evokes severe aberrant phenotypes in clover root hair development, disrupting the balance between biosynthesis and degradation of its cell wall.

Because localized regions of nodular infection threads that develop into infection droplets exhibit a non-crystalline isotropic cell wall (Robledo et al., 2011a), we predicted that CelC2 may be involved in infection droplet development within nodule cells. To examine if CelC2 has a symbiotic function during this event of the secondary infection process, we monitored both ANU843wt and CelC2-overexpressing strains behavior within nodules by transmission electron microscopy (TEM), illustrated in Figure 53.3.

ANU843wt strain followed the normal steps of the infection process: dissemination of bacteria within walled infection threads within plant cells, localized hydrolysis of the infection thread wall, movement of the vegetative bacteria into membrane-enclosed infection droplets that protrude into the host cell cytoplasm, and endocytosis of bacteria from infection droplets followed by their differentiation into nitrogen-fixing competent bacteroids, forming the nitrogen-fixing symbiosomes within host nodule cells (Fig. 53.3a).

Secondary host infection by the recombinant strain ANU843C2+ included steps that were similar to and others that were different from wild type ANU843. Bacterial progression through the infection thread was similar to wild type, except for more frequent areas of localized hydrolysis resulting in an increase in the diameter of the lumen of infection threads, and infection droplets that were larger with prominent multilobed protrusions (Robledo et al., 2011a). Often, vegetative cells of the CelC2 over-producing strain were released into the host cells without being surrounded by a peribacteroid membrane or bacteroid differentiation (Fig. 53.3b). The cell wall of nodule cells containing naked vegetative bacteria showed significant deformation accompanied by thinning and breakage indicating significant disruption of this host barrier (Robledo et al., 2011a). Thus, invasion of host nodule cells by the CelC2 over-producing strain resulted in uncontrolled disorganization of the plant

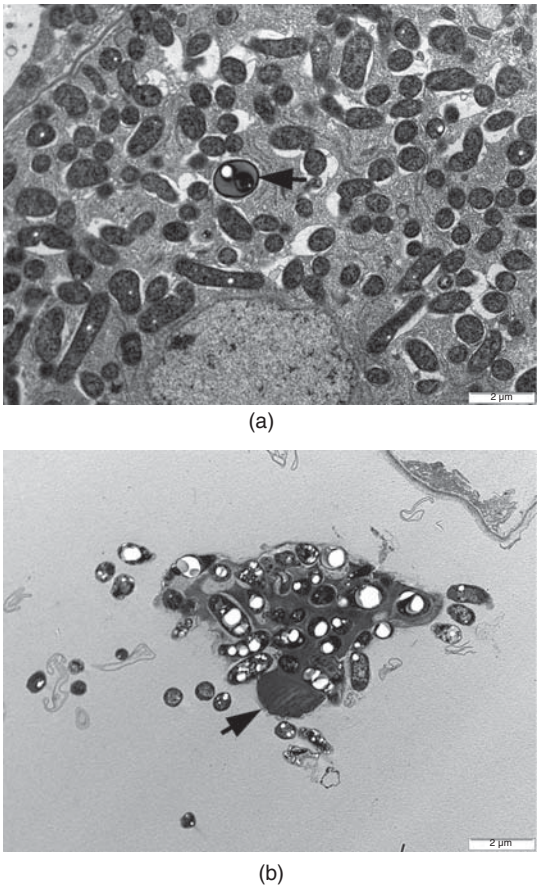

Figure 53.3 Effect of *Rhizobium* cellulase CelC2 overproduction on secondary infection within white clover root nodules. TEM images showing clover nodule cells in the infection zone inoculated with ANU843 (a) or ANU843C2+ (b). Infection threads are marked with arrows.

cell wall and absence of well-defined symbiosome formation, leading to premature senescence of nodules that do not fix nitrogen (Robledo et al., 2011a).

These observations indicate that the CelC2 cellulase could intervene in localized erosion of and bacterial liberation from infection threads within the nodule, and that its *in situ* concentration and activity must be critically regulated to advance successful symbiotic development. Thus, an excess of CelC2 production leads to an aberrant, exaggerated Bar phenotype of bacterial release without peribacteroid membrane enclosure, followed by the induction of nodule premature senescence and eventual abortion of the symbiotic nitrogen-fixing process (Robledo et al., 2011a).

53.4 CONCLUSIONS

Our results support: (i) a key role of the rhizobial CelC2 cellulase in cellulose biosynthesis by modulating the length of the cellulose fibrils that mediate firm adhesion among *Rhizobium* bacteria leading to biofilm formation; (ii) the importance of *Rhizobium* cellulose as an essential component of the biofilm polysaccharidic matrix architecture; (iii) the essential role of rhizobial CelC2 cell wall-degrading enzyme in the primary infection process; and (iv) its importance in secondary symbiotic infection and tight regulation of its production to establish an effective nitrogen-fixing root nodule symbiosis.

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Chapter 54

Nod Factor-Induced Calcium Signaling in Legumes

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54.1 INTRODUCTION

In plants, calcium is a ubiquitous signal molecule involved in a number of developmental processes including pollen tube and root hair apical growth, regulation of stomatal aperture, and responses to abiotic and biotic stresses (Foreman et al., 2003; Feijo et al., 2001; McAinsh et al., 1995; Dodd et al., 2010). A calcium signal can consist of a single elevation in calcium within a cellular compartment, for example, the sustained elevations of calcium in the cytoplasm and nucleus caused by the recognition of pathogen-associated molecular patterns (PAMPs) during innate plant defense (Dodd et al., 2010). They can also exhibit oscillatory behavior, such as the tip-focused calcium gradients in pollen tubes and root hair cells, which oscillate for the same period as the oscillations in cell growth (Feijo et al., 2001; Monshausen et al., 2008). In *Arabidopsis thaliana* root hairs the oscillations in calcium have a period of about 30 s but calcium oscillations can have a much longer period; for example, oscillations with a period of 24 h are involved in circadian clock regulation (Love et al., 2004). During the establishment of the legume-rhizobial symbiosis (Sym), rhizobia produce lipo-chitooligosaccharides (LCOs) known as Nod factors (NFs). NFs are first perceived by the legume at the root epidermis and are capable of activating two different calcium responses: nuclear-localized calcium oscillations (spiking) and a tip-focused calcium influx. These two calcium responses are associated with the activation of nodule organogenesis and the initiation of bacterial infection.

54.2 CALCIUM SPIKING

Upon NF treatment there is a delay of 10–15 min before calcium spiking starts around the nuclear region of legume root hair cells (Ehrhardt et al., 1996; Miwa et al., 2006b). Use of both nuclear-targeted and nuclear-excluded calcium reporters confirms that the spiking originates from the periphery of the nucleus and can be observed both in the nucleoplasm and the nuclear-associated cytoplasm, suggesting that the nuclear envelope and nuclear-associated endoplasmic reticulum (ER) are the calcium sources (Sieberer et al., 2009; Capoen et al., 2011). The response can be activated by NF concentrations as low as 10^{-12} M and can also be activated by chitin oligomers, NF analogs, and mycorrhizal-produced Lipochitooligosaccharides (Myc-LCOs) (Oldroyd et al., 2001a; Walker et al., 2000; Genre et al., 2013). Once activated by NF, calcium spiking is very robust and can be observed in a root hair cell for many hours (Walker et al., 2000; Miwa et al., 2006a). In the model legume *Medicago truncatula* the spikes have a period of around 90 s, although this can vary over the course of the spiking response and between cells (Kosuta et al., 2008; Sun et al., 2007).

Calcium spiking is central to the common Sym signaling pathway (also known as CSSP) required to form symbioses with rhizobia and mycorrhizal fungi (Fig. 54.1). The pathway has a core set of genetic components required for both symbioses that are involved with generating or "encoding" the calcium spiking response and "decoding" it to lead to the activation of downstream genes. Downstream of

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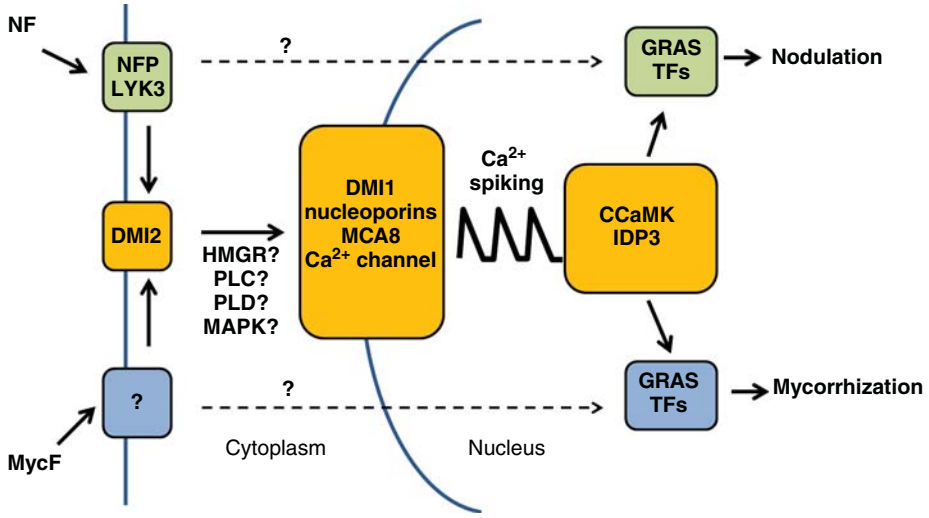

Figure 54.1 The Sym pathway. The Sym pathway (shown in orange) is shared between nodulation and mycorrhization. NFs from rhizobia are perceived by NFP and LYK3 at the plasma membrane and mycorrhization factors (MycFs) are perceived by currently unidentified receptors. The signals from both symbionts activate DMI2 also located at the plasma membrane and then nuclear calcium spiking is mediated by the cation channel DMI1, nucleoporins, the Ca^{2+}-ATPase MCA8 and an unidentified calcium channel. Downstream of calcium spiking CCaMK is activated and with its interacting partner IPD3 promotes appropriate gene expression for either nodulation or mycorrhization via activation of GRAS transcription factors (TFs). Parallel signaling may also be involved.

calcium decoding differential transcription factors (TFs) activate genes required for nodule formation and bacterial infection in the rhizobial Sym, or to facilitate fungal infection during the arbuscular mycorrhizal (AM) Sym. During nodulation, genes induced by the Sym pathway include *ENOD11* and the TFs *NIN* and *ERN* (Journet et al., 2001; Marsh et al., 2007; Schauser et al., 1999; Middleton et al., 2007).

In *Lotus japonicus* calcium spiking is dependent on the NF receptors NFR5 and NFR1 of the LysM receptor kinase-like family (Miwa et al., 2006b). In *M. truncatula* the homologs are Nod factor perception (NFP) and LYK3, but interestingly only NFP is required for NF-induced calcium spiking (Smit et al., 2007; Ben Amor et al., 2003). Another plasma membrane component, a leucine-rich repeat (LRR)-receptor kinase called DMI2/SymRK (does not make infections 2) is required for NF induction of calcium spiking and is required for both rhizobial and mycorrhizal Sym (Wais et al., 2000; Endre et al., 2002; Stracke et al., 2002). It is likely that these receptors act in a complex to bind NF (ref) and activate downstream signaling.

54.3 SECONDARY MESSENGERS: LINKING NF RECOGNITION AT THE PLASMA MEMBRANE WITH NUCLEAR CALCIUM SPIKING

From the plasma membrane the signal generated by NF recognition must be relayed to the nucleus probably by secondary messengers to generate calcium spiking, but the mechanism for this is unclear. There is evidence for the involvement of phospholipase C (PLC) and phospholipase D (PLD) in the Sym pathway. IP_3 along with diacylglycerol (DAG) are the products of phosphatidylinositol 4,5-bisphosphate (PIP-2) hydrolysis by PLC and the PLC inhibitor U73122 blocks NF-induced calcium spiking and pENOD11-GUS expression (Pingret et al., 1998; Engstrom et al., 2002). PLD can catalyze the conversion of DAG to phosphatidic acid, which can also act as a signal molecule. NF activates PLD activity in legumes (den Hartog et al., 2001, 2003) and n-butanol treatment inhibits calcium spiking and pMtENOD11-GUS expression suggesting that PLD may also be involved in the Sym pathway (Charron et al., 2004; Sun et al., 2007).

Interestingly, the G-protein agonist mastoparan (or its synthetic analog Mas7) can activate calcium oscillations similar to NF-induced calcium spiking and can induce pMt-ENOD11-GUS expression (Sun et al., 2007; Pingret et al., 1998). However, there are some differences in NF and Mas7-induced calcium spiking signatures. Mas7 leads to calcium oscillations with a slower initial release of calcium and greater period variability and the oscillations are not confined to the nucleus but can be observed all over the cell (Sun et al., 2007). Unlike the NF response it is independent of *NFP* and *DMI2*, suggesting that it either acts downstream of these receptors or by an independent mechanism. Several G-proteins have been identified as having roles in nodulation including members of the rho of plants guanosine triphosphatase (ROP GTPase) family and a Rab GTPase (Blanco et al., 2009; Ke et al., 2012). *L. japonicus* ROP6 is able

to bind to NFR5 and the gene is expressed in nodules and infection threads after inoculation with *Mesorhizobium loti* (Ke et al., 2012). In *ROP6* RNA interference (RNAi) lines infection thread development into the cortex is impaired and few nodules are formed (Ke et al., 2012). G-proteins can activate multiple signaling pathways including IP_3 production and in pollen tube tips PIP-2 accumulation is dependent on ROP-GTPases (Kost et al., 1999). Mastoparan activates PLC and PLD in the legume *Vicia sativa* (den Hartog et al., 2001). Altogether these results suggest that NF receptor activation could result in the activation of ROP-GTPases leading to the production of IP_3 and DAG to activate calcium spiking in the nucleus. However, there is no direct evidence for IP_3 induction of calcium spiking and mastoparan-induced calcium release during the activation of plant mitogen-activated protein kinase (MAPK) signaling occurs independently of G-proteins (Miles et al., 2004) so care should be taken in interpreting these Mas7 results.

Multiple secondary messengers may be interacting in a signaling pathway so even if IP_3/DAG are involved in generating NF-induced calcium spiking it is possible that other secondary messengers are also required. A MAPK kinase SIP2 interacts with DMI2/SymRK (Chen et al., 2012). In *L. japonicus* SIP2 RNAi plants produced fewer nodules and three nodulation marker genes including *NIN* were downregulated suggesting that an MAPK signaling cascade may be involved in generating calcium spiking. An enzyme known as HMGR1 (3-hydroxy-3-methylglutaryl coenzyme A reductase 1) also interacts with MtDMI2 (Kevei et al., 2007). HMGRs catalyze the production of mevalonate, a precursor required for isoprenoid biosynthesis. Treatment with lovastatin, an HMGR inhibitor, leads to reduced numbers of nodules in *M. truncatula* plants inoculated with *Sinorhizobium meliloti*, and *MtHMGR* RNAi lines produce fewer nodules than the wildtype suggesting that mevalonate is required for nodule formation. It may be that mevalonate is required for the production of isoprenoid compounds such as cytokinins or phytosteroids but it is also possible that mevalonate or a downstream compound is acting as a secondary messenger to relay the NF signal to the nucleus to activate calcium spiking.

54.4 ENCODING CALCIUM SPIKING: THE NUCLEAR ENVELOPE MACHINERY

Once the NF signal reaches the nucleus there are a number of genes that are required to induce calcium spiking. *DMI1* encodes a protein that localizes preferentially to the inner nuclear membrane with structural similarity to the pore domain of MthK, a calcium-activated potassium channel in *Methanobacterium thermoautotrophicum* (Ane et al., 2004; Edwards et al., 2007; Imaizumi-Anraku et al., 2005; Riely et al., 2007; Wais et al., 2000; Charpentier et al., 2008; Venkateshwaran et al., 2008; Capoen et al., 2011; Miwa et al., 2006b). In *L. japonicus* there are two homologs of *DMI1* required for calcium spiking known as *CASTOR* and *POLLUX* (Charpentier et al., 2008). CASTOR has ion channel activity *in vitro* with preferential mobilization of potassium, and POLLUX can complement a potassium-transport-deficient yeast mutant, providing evidence that they are functional potassium-permeable channels (Charpentier et al., 2008). Although *M. truncatula* has a gene orthologous to *CASTOR* it is not required for calcium spiking. *L. japonicus castor* and *pollux* mutants can be complemented by *DMI1* suggesting that *DMI1* in *M. truncatula* can fulfill the roles of both *CASTOR* and *POLLUX* (Venkateshwaran et al., 2012). *NUP85*, *NUP133*, and *NENA* encode subunits of the nucleopore and are also required for NF induction of calcium spiking. These proteins possibly function in the transport of membrane proteins to the inner nuclear membrane (Kanamori et al., 2006; Saito et al., 2007; Groth et al., 2010), and obvious substrates would be DMI1, POLLUX and CASTOR.

For the initiation of a calcium spike, where the calcium concentration in the nucleus and the nuclear-associated cytoplasm increase calcium permeable channels must be present in the nuclear membrane to allow calcium release from the nuclear periplasm. The genetic identity of the channel is still unknown. Its absence among nodulation mutants identified in several forward genetic screens conducted over the last two decades suggests that there may be multiple different genes involved or they may have other roles in development.

To return the calcium released into the nucleus and the nuclear-associated cytoplasm back to its store, a calcium pump is required that actively transports calcium across the nuclear membrane against its electrochemical gradient. A role for plant type IIA (SERCA-type) Ca^{2+}-ATPases has been suggested by an inhibitor cyclopiazonic acid and two activators gingerol and butylated hydroxynanisole that can block calcium spiking (Capoen et al., 2009, 2011; Engstrom et al., 2002). RNAi of a nuclear-localized SERCA Ca^{2+}-ATPase (MCA8) results in a large reduction in the number of root hair cells with NF-induced calcium spiking (Capoen et al., 2011). MtMCA8 is localized to the nuclear membranes, but has no preference for the inner or outer membranes. This location coupled with the phenotype of RNAi suggests that MCA8 acts to return calcium to the nuclear envelope during NF-induced calcium spiking.

54.5 MODELING CALCIUM SPIKING

To further understand how the NF-induced calcium oscillations in the nucleus may be generated Granqvist and colleagues (2012) have developed a mathematical model

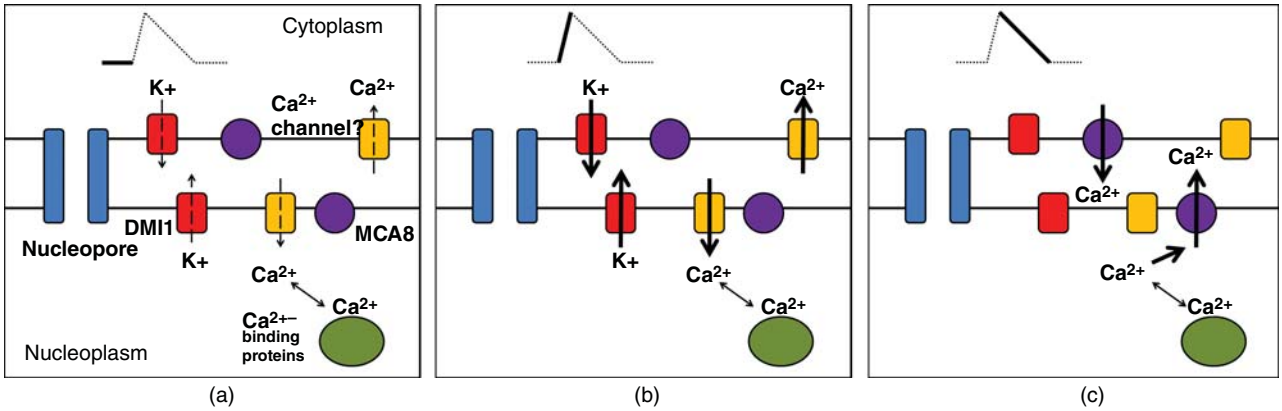

Figure 54.2 A model for calcium spiking. Model of calcium spike generation at the nuclear membranes. (a) Prior to the start of a calcium spike the membrane potential is negative on the nucleoplasmic side close to the potassium resting potential and DMI1 (shown in red) is weakly conducting potassium. This drives a transient influx of calcium ions through the calcium channel (orange) into the nucleoplasm leading to the depolarization of the membrane until it reaches the calcium resting potential. (b) The conductivity of DMI1 increases, resulting in the flow of potassium ions down their electrochemical gradient into the nuclear lumen. This hyperpolarizes the inner nuclear membrane, generating the large calcium flow into the nucleoplasm that forms the upwards part of the calcium spike. (c) Once the membrane returns to the potassium ion resting potential the potassium and calcium currents almost cease and the downwards part of the calcium spike is formed by the action of MCA8 (purple) returning calcium ions to the nuclear envelope lumen. Since the electrochemical gradient of calcium is so large once the membrane potential returns to the starting value the conductance of the voltage-activated calcium channel increases leading to a transient release of calcium that restarts the cycle. The frequency and shape of calcium spikes can be modulated by altering concentrations of calcium-binding proteins (green) in the nucleus.

to simulate the oscillations (Fig. 54.2). It consists of three membrane components: a Ca^{2+}-activated K^+ channel (based on DMI1), a voltage-gated calcium channel and a calcium-pump (based on MCA8). Assuming that the nuclear membrane potential is more negative on the nucleoplasmic side than in the nuclear envelope lumen the model can produce self-sustaining calcium oscillations in the nucleoplasm in the simulation similar to those experimentally observed in *M. truncatula*. However, the calcium oscillations in this model continue indefinitely, suggesting that additional components or activities are required for the cessation of the response.

The addition of Ca^{2+}-binding proteins, which act as Ca^{2+} buffers, improves the ability of the model to simulate calcium spiking. By altering buffer concentrations they can stop the oscillations or alter the period of the oscillations to simulate the observed variation in experimental data. When the model was set up with high levels of unbound calcium-binding proteins at the start, rapid calcium oscillations preceded the regular spiking pattern. This is very similar to experimental observations where, in about 50% of cases calcium spiking appeared to start with a rapid oscillatory phase before the regular spiking pattern can be established (Granqvist et al., 2012). Experimentally adding additional NF after the induction of calcium spiking leads to a short stage of rapid oscillations before the regular spiking pattern resumes in *M. truncatula* root hair cells, suggesting that NF may increase calcium buffering capacity in the nucleus by regulating calcium-binding proteins.

While the model (Fig. 54.2) can simulate calcium oscillation it is not without its limitations. For simplicity only the nucleoplasm and the nuclear membrane lumen are included and within those compartments it does not include any spatial information. Nevertheless, it proposes a mechanism for how a relatively small number of genetic components can generate robust calcium spiking and predicts that calcium-binding proteins are important modulators. It indicates that the currently unidentified calcium channel could be voltage-gated, although a ligand-gated or a dual-regulated channel could also be possible.

The function of the cation channel DMI1 can also be hypothesized using the model. It could be acting to counter balance the flow of the positively charged calcium ions into the nucleoplasm when calcium channels are activated, or alternatively it could directly trigger the activation of a voltage-gated calcium channel by polarizing the inner nuclear membrane potential. Charpentier and colleagues (2013) found that calcium spiking is only initiated when DMI1 and the calcium channel are activated simultaneously with DMI1 conductance dependent on the membrane potential and calcium concentration (Fig. 54.3). It indicates that although the main function of DMI1 is to act as a counter-ion channel, initial low-level potassium ion conductance is required to activate a transient calcium ion flux. In turn, this activates both DMI1 and the calcium channel by depolarization of the nuclear membrane. The conclusions from the model are supported by the following evidence: the homology of DMI1 to a calcium-activated

potassium channel, DMI1 interferes with galactose-induced cytosolic calcium transient increases in yeast cells and when expressed in human embryonic kidney (HEK) cells is sufficient to drive calcium-induced calcium release (Edwards et al., 2007; Venkateshwaran et al., 2008; Peiter et al., 2007). All this indicates the ability of DMI1 to coordinate calcium channels in diverse cell types. From the model it seems possible that the calcium spiking machinery could be primed for activation even in the absence of NF, suggesting that DMI1 or the calcium channel may be negatively regulated and NF perception releases them enabling calcium spiking to be initiated.

It is still not clear whether the calcium spiking in the nucleoplasm and the nuclear-associated cytoplasm is a consequence of calcium fluxes across both the inner and outer nuclear membrane, or is due to calcium fluxes across only one side with calcium ions flowing from the nucleoplasm to the cytoplasm or *vice versa* through nuclear pores to generate the spiking response on the other side. So far, using current imaging techniques, it has not yet been possible to distinguish between these scenarios (Capoen et al., 2011). However, modeling suggests that diffusion through the nuclear pore is insufficient to explain the calcium release inside and outside the nucleus. Calcium spiking in the nucleoplasm has a clear function because this is where the downstream components of the Sym pathway including the calcium/calmodulin-dependent kinase (CCaMK) and TFs such as NSP1 and NSP2 are located (Riely et al., 2007; Mitra et al., 2004; Hirsch et al., 2009; Kalo et al., 2005; Levy et al., 2004). The question remains whether calcium spiking in the nuclear-associated cytoplasm is just a consequence of the presence of machinery on the inner and outer nuclear membranes and/or the permeability of calcium through nuclear pores, or whether it has a biologically relevant function in nodulation.

54.6 DECODING CALCIUM SPIKING IN THE NUCLEUS

Mutants defective for CCaMK retain calcium spiking but lack NF-induced gene expression and nodulation suggesting that CCaMK is positioned downstream of calcium spiking (Levy et al., 2004; Mitra et al., 2004; Miwa et al., 2006b) and likely acts to decode this signal. CCaMK has a kinase domain at the N-terminal end, a calmodulin (CaM)-binding domain/autoinhibitory domain and three calcium-binding EF hand motifs, making it highly unusual as it is capable of perceiving both free calcium ions and calcium bound to CaM (Patil et al., 1995) (see Fig. 54.3). The kinase and CaM binding domains have sequence similarity to calmodulin-dependent protein kinase II (CaMKII) in animals, which is capable of frequency dependent activation by calcium, making CCaMK a good candidate for the decoding of calcium spiking (De Koninck and Schulman, 1998; Patil et al., 1995).

There are several gain-of-function mutations in CCaMK, including truncated "kinase only" and point mutations of the threonine at position 271 in *M. truncatula* (or equivalent in other species), which result in the activation of nodulation gene expression and spontaneous nodule formation even in the absence of rhizobia (Gleason et al., 2006; Hayashi et al., 2010; Takeda et al., 2012; Tirichine et al., 2006). The gain-of-function mutations demonstrate that CCaMK activation is sufficient to activate downstream components of the Sym pathway and also that T271 has a crucial role in the regulation of the protein. CCaMK kinase activity can be regulated by autophosphorylation and mutations of the equivalent threonine residue in *Lilium longiflorum* CCaMK resulted in lower levels of autophosphorylation and substrate phosphorylation, supporting a role for T271 in regulation (Sathyanarayanan et al., 2001). Expression of autoactive CCaMK T265D and T265I (equivalent to T271) variants in *L. japonicus* Sym pathway mutants that are unable to produce calcium spiking, restored nodulation, and AM colonization demonstrating that the primary purpose of generating nuclear calcium spiking is the activation of CCaMK (Hayashi et al., 2010; Madsen et al., 2010). However, the infection process was delayed and was less efficient with fewer functional nodules, probably due to deregulated CCaMK activity and ectopic expression, especially in the case of $CCaMK^{T265D}$, which was expressed under control of a constitutive promoter.

The two calcium-sensing domains appear to play different roles in the activation of CCaMK. CaM binding to CCaMK decreases phosphorylation of CCaMK (Takezawa et al., 1996). The autoinhibitory domain of CCaMK overlaps with the CaM-binding domain, so CaM-binding may release autoinhibition of the protein (Patil et al., 1995). The calcium-binding affinities to CaM and that of CaM binding to CCaMK suggest that CaM binding to CCaMK may only occur at significant levels at the higher concentrations of calcium generated by calcium spiking (Swainsbury et al., 2012). In contrast, the affinities of calcium binding to the EF hands suggests that at basal calcium concentrations before calcium spiking starts some of the EF hands may be occupied by calcium. The EF hands could inhibit CCaMK activation at low calcium concentrations or perhaps prime CCaMK for activation by CaMbinding or both. Therefore, the EF hands and the CaM-binding domain could provide CCaMK with mechanisms for sensing both high and low levels of calcium to facilitate the switch from the inactive to active state during calcium spiking (Fig. 54.3).

Point mutations in T271 and the CaM-binding domain of CCaMK have provided some insights into the mechanism of activation of the protein. Interestingly, both phospho-ablative (T-A) and phospho-mimic (T-D) mutations in T271 result in spontaneous nodule formation in legumes (Gleason

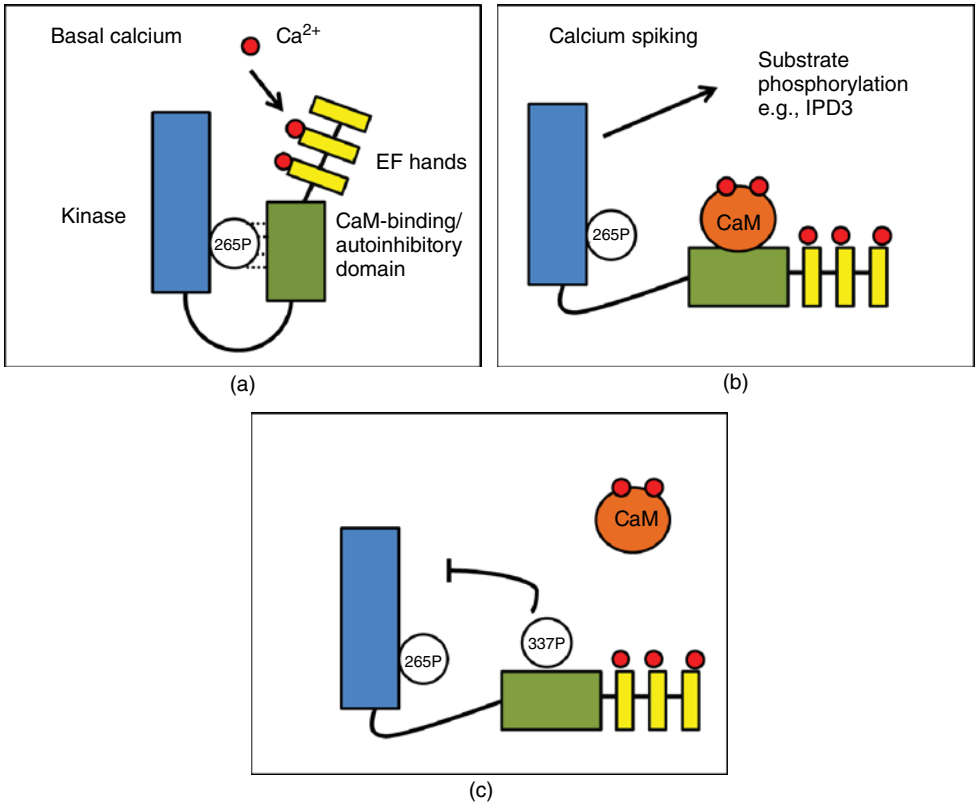

Figure 54.3 Model of CCaMK regulation. (a) Inactive CCaMK with a hydrogen bond network linking the kinase and CaM-binding/autoinhibitory domains. At basal calcium concentrations some of the EF hands are occupied by calcium ions, promoting Threonine 265 phosphorylation to drive the hydrogen-bond network, deactivating CCaMK. (b) At the onset of calcium spiking higher calcium concentrations result in full occupancy of the EF hands and calcium-bound to calmodulin (CaM). CaM binds to the CaM-binding domain, inducing a conformational change that releases the inhibition of the kinase domain, resulting in phosphorylation of CCaMK targets (e.g., IPD3/CYCLOPS). (c) Inactivation of CCaMK by phosphorylation of serine 337 in the CaM-binding domain, which prevents CaM binding and restores autoinhibition of the kinase domain by association with the CaM-binding/autoinhibitory domain.

et al., 2006; Takeda et al., 2012; Tirichine et al., 2006). A possible explanation of how these "opposite" mutations could have the same phenotypes has been provided by homology modeling of CCaMK with *Caenorhabditis elegans* CaMKII (Shimoda et al., 2012). This homology modeling predicts that in *L. japonicus* CCaMK residue T265 forms a hydrogen bond network with neighboring residue R317 in the CaM-binding/autoinhibitory domain. According to the model, mutation or phosphorylation of T265 would disrupt the hydrogen bond network, weakening the link between the kinase and CaM-binding/autoinhibitory domain, thus preventing effective autoinhibition even in the absence of calcium (Shimoda et al., 2012). It is possible that calciumbinding to the EF hands at low calcium concentrations may facilitate autophosphorylation of T271 to prime CCaMK for activation by CaMbinding at higher calcium concentrations (Fig. 54.3).

CCaMK can be negatively regulated by phosphorylation of a residue in the CaM-binding domain (Fig. 54.3). The *ccamk-14* mutant in *L. japonicus* has a serine to asparagine mutation at position 337 (Liao et al., 2012). The mutant is defective in mycorrhizal infection and during rhizobial infection produces more infection threads in the epidermis but the infection threads are impaired in progression through to the cortex, although nodule formation is unaffected. The phospho-mimic variant $CCaMK^{S337D}$ is unable to complement *ccamk-1*, suggesting that $CCaMK^{S337D}$ is not active. These mutations also indicate that negative regulation of CCaMK is required to facilitate bacterial infection, but is not required for nodule development or mycorrhizal infection.

CCaMK interacts with phosphorylates IPD3 (CYCLOPS in *L. japonicus*), a protein of unknown function with two nuclear localization signal motifs and a C-terminal coiled-coil motif thought to mediate protein–protein interactions (Messinese et al., 2007; Yano et al., 2008). *IPD3/CYCLOPS* is required for both rhizobial and AM infection (Yano et al., 2008). Although the mechanisms are currently unclear, CCaMK/IPD3 complex activation and recruitment of several GRAS TFs including *NSP1*, *NSP2*,

and *RAM1* are involved in the activation of specific nodulation or mycorrhizal gene expression (Heckmann et al., 2006; Kalo et al., 2005; Smit et al., 2005; Gobbato et al., 2012).

54.7 DO CALCIUM SPIKING SIGNATURES ENCODE SPECIFICITY BETWEEN NODULATION AND MYCORRHIZATION?

One of the most interesting questions regarding Sym pathway function is how activation of a shared signaling pathway can result in the differential outputs required to establish either the rhizobial or mycorrhizal Sym. Either specificity must be encoded within the pathway itself or a parallel pathway exists that modulates the output of the Sym pathway to induce the appropriate changes in gene expression. If specificity is encoded within the pathway it would be expected that the calcium spiking signatures would be different between the symbioses and CCaMK would be capable of differential activation, as is CaMKII in animal systems (De Koninck and Schulman, 1998).

The concept of different calcium spiking signatures is supported by mathematical analyses of calcium spiking signatures in *M. truncatula* produced by NFs and the mycorrhizal fungus *Rhizophagus irregularis* (previously, *Glomus intraradices*). Kosuta and colleagues (2008) found that the duration of a mycorrhizal-induced calcium spike is shorter than an NF-induced spike and there are also differences in shape. However, this experimental set up was not optimal because when using the fungus it is impossible to determinate when the plant root first perceives the fungus and the concentrations of the then unidentified diffusible signal factors dubbed "Myc factors." Using a different experimental set up Sieberer and colleagues (2012) studied calcium spiking signatures during the progression of infection of rhizobia and mycorrhizal fungi (see also Chapter 57). They found that for both symbioses, cortical cells switched from low frequency calcium oscillations prior to the microbes reaching the cell to high frequency oscillations during apoplastic entry. The high frequency oscillations appeared to be similar in both symbioses so it seems likely during the infection process that atleast calcium oscillations do not encode symbiont specificity. Myc-LCOs and chitin oligomers from fungal exudates that are capable of activating calcium spiking have recently been identified and if mathematical analyses of these calcium spiking signatures with those produced by NFs is carried out it may lead to some clarification (Genre et al., 2013; Maillet et al., 2011).

Instead of spike duration and shape encoding specificity CCaMK could be differentially activated by the number of calcium spikes that occur. In *M. truncatula* NF-induced calcium spiking only leads to *ENOD11* expression after about 36 individual spikes have occurred (Miwa et al., 2006a). It could be possible that during AM colonization a different number of calcium spikes occur, perhaps due to differences in nuclear Ca^{2+}-binding protein concentrations that terminate spiking sooner or later than during nodulation.

CCaMK activation does not appear to be associated with differential induction of symbiotic-specific responses. The autoactive $CCaMK^{1–314}$ (kinase only) variant activated both the nodulation marker *NIN* and the mycorrhizal marker subtilase (*SbtM1*) expression when expressed in *L. japonicus* (Takeda et al., 2012). Interestingly, *SbtM1* is expressed in multiple cell layers in $CCaMK^{1–314}$ expressing plants and is associated with the formation of fungal infection-like structures, whereas *NIN* expression is restricted to the epidermis (Takeda et al., 2012). However, the *snf1* mutant, which expresses autoactive $CCaMK^{T265I}$ induced nodulation-specific gene expression but not AM-specific gene expression, indicating that the phosphorylation status of threonine 265 may be important in activating rhizobial-associated responses (Takeda et al., 2011).

At this stage it seems unlikely that differences in calcium spiking responses can allow CCaMK to discriminate between the rhizobia and AM fungi and, instead, alternative signaling may define the specificity of the response.

54.8 THE CALCIUM INFLUX

In addition to calcium spiking, NF treatment also induces a calcium influx into the cytoplasm that originates in the tip of root hair cells (Miwa et al., 2006b; Shaw and Long, 2003a; Walker et al., 2000). Using ion-selective electrodes, Felle and colleagues (1998) were able to detect the calcium influx occurring within seconds of NF addition with calcium ions moving into the cytoplasm from the extracellular matrix. To initiate the calcium influx response, higher concentrations of NF must be used than those required to initiate calcium spiking, with 10^{-9} M required for half maximal induction and 10^{-8} M typically used to induce the response reliably (Felle et al., 2000; Miwa et al., 2006b; Shaw and Long, 2003a). Non-sulfated NFs and chitin oligomers, which are able to activate calcium spiking, do not activate the calcium influx suggesting that it has higher NF stringency (Felle et al., 1999; Miwa et al., 2006b; Shaw and Long, 2003a; Walker et al., 2000). In addition to being temporally and spatially separate it is also possible to genetically separate calcium spiking and the calcium influx. Both responses are dependent on the NF receptor *NFP* (in *L. japonicus NFR5* and *NFR1*) but the sym pathway mutants *dmi1* and *dmi2* both retain the calcium influx response (Ben Amor et al., 2003; Miwa et al., 2006b). Altogether these results suggest that the calcium influx is involved in a parallel pathway independent of calcium spiking. However, Shaw and Long (2003a) reported that the calcium influxes observed in *dmi1*

and *dmi2* after NF addition appeared to be shorter in duration than in wildtype *M. truncatula*, so it is possible that *DMI1* and *DMI2* can modulate the calcium influx.

The requirement for higher concentrations of NF and the higher stringency suggest that calcium influx may be involved in bacterial infection (Miwa et al., 2006b). In the early stages of establishment of the Sym before rhizobia attach to the root surface, the NF concentrations perceived by the plant are likely to be low, but presumably high enough to activate calcium spiking to induce early nodulation gene expression. During infection rhizobia attach to the surface of the tips of root hair cells, inducing the root hair to curl around the rhizobia entrapping them in infection foci where they continue to divide. The next stage is the development of the infection thread, a plant-produced structure that guides the dividing rhizobia down through the root hair into the root cortex to enable the bacteria to infect the developing nodule. Throughout this process as the rhizobia come into closer contact with the plant cell membranes and the number of "trapped" bacteria increase, the local NF concentration at the plant membranes is expected to increase, perhaps to a threshold where the calcium influx might be activated, and this may be associated with root hair curling or infection thread initiation. The infection process also has more stringent requirements for NF than induction of the Sym pathway, demonstrated by the *nodO/nodE* double mutant of *Rhizobium leguminosaurum* bv. v*iciae*, which induces root hair deformation and many infection foci on vetch but rarely forms infection threads (Walker and Downie, 2000). The *NodO* gene encodes a secreted protein that is capable of forming cation-selective pores in membranes (Sutton et al., 1994) and restoration of it or *NodE* (involved in attachment of acyl groups to NF) rescued infection thread development in the mutant, suggesting that an ion flux across the plant membrane induced by NodO could compensate for defective NF structures.

54.9 A ROLE FOR THE APYRASE LNP IN NF-INDUCED CALCIUM SIGNALING

Calcium spiking and the calcium influx are both dependent on *Lectin Nucleotide Phosphohydrolase (LNP)*. *LNP* antisense lines in *L. japonicus* do not form nodules or infection threads and the lines are defective for calcium spiking and the calcium influx, but maintain root hair deformation (Roberts et al., 2013). LNP has apyrase (hydrolysis of phophoanhydride bonds of nucleoside triphosphates and diphosphates) activity and is able to bind NF (Etzler et al., 1999, 2000). Extracellular adenosine triphosphate (ATP) is a signal molecule involved in a wide range of processes in plants and both ATP and ADP (adenosine diphosphate) are able to induce increases in cytosolic free calcium (Roux and Steinebrunner, 2007; Demidchik et al., 2003, 2009, 2011; Jeter et al., 2004). ADP treatment partially rescued Soybean LNP (GS52) RNAi lines, which are defective for nodule development and infection, suggesting that ADP may have a role in promoting nodulation (Govindarajulu et al., 2009). Perhaps, during nodulation, LNP regulates extracellular ADP/ATP levels to modulate calcium responses (For a more detailed review see Chapter 52: The Role of Apyrases in Nodulation.).

54.10 THE CALCIUM INFLUX IS SPATIALLY AND TEMPORALLY COINCIDENT WITH OTHER NF RESPONSES

After NF addition, there is an efflux of chloride ions, intracellular alkalization, and plasma membrane depolarization at the tip of a legume root hair cell, all occurring a few seconds after the calcium influx (Ehrhardt et al., 1992; Felle et al., 1996, 1998; Kurkdjian, 1995). The calcium inhibitor nifedipine inhibits these responses and the responses can be mimicked by treatment with the calcium ionophore A32187 and the Ca^{2+}-ATPase inhibitor 2,5-di(t-butyl)-1,4-benzohydroquinone (BHQ), providing further evidence that the calcium influx acts upstream of these responses (Felle et al., 1998). After membrane depolarization there is an efflux of potassium ions from the cell and membrane repolarization. Similarly to the calcium influx, the membrane depolarization is not induced by non-sulfated NFs or chitin oligomers and is dose dependent with a maximal response produced at $10^{-8}/10^{-7}$ M NF (Ehrhardt et al., 1992; Felle et al., 1996; Shaw and Long, 2003a). Felle and colleagues (1998) suggested a model where the NF-induced calcium influx acts as a secondary messenger leading to the activation of the chloride efflux and intracellular alkalinization, perhaps by inhibition of proton pumps, to generate the membrane depolarization. They also suggested that membrane repolarization is achieved by the efflux of potassium ions through potassium permeable channels, which is activated once the membrane potential becomes more positive than the potassium equilibrium potential.

Other responses to NF also occur at the tip. Within 3–6 min of NF addition cytoskeletal changes occur in legumes (Cardenas et al., 1998; Weerasinghe et al., 2003). The cytoskeletal changes precede NF-induced root hair deformation. There is also a transient increase in reactive oxygen species (ROS) production starting around 1 min after NF addition with ROS levels restored to normal levels by around 3 min (Cardenas et al., 2008; Shaw and Long, 2003b). Chitin oligomers were unable to produce the response, and the fungal elicitor chitin produced a larger, more sustained increase in ROS production more like the ROS burst seen during PAMP recognition in plant defence. Pretreatment of

root hair cells with DPI (diphenyleneiodonium), an inhibitor of NADPH oxidases and other flavin-containing enzymes, inhibited the response, suggesting that the main source of the ROS may be from NADPH oxidases (Cardenas et al., 2008). During the apical growth of root hair cells and pollen tubes calcium, protons and ROS interact to drive polar growth so it is possible that during nodulation the transient ROS production may be involved with the calcium influx and intracellular alkalinization that lead to membrane depolarization (Foreman et al., 2003; Monshausen et al., 2007, 2008). Although the ROS transient appears to be occurring later than the calcium influx and membrane depolarization, these processes have not been measured concurrently and the differences may be associated with technical differences in the experiments.

ROS can act as signal molecules in plants and there is evidence that ROS levels are regulated throughout the nodulation process (Apel and Hirt, 2004). Increased superoxide production can be detected in infection threads formed during the *M. sativa-S. meliloti* Sym, and there is an accumulation of hydrogen peroxide during nodule development and bacterial infection (Rubio et al., 2004; Santos et al., 2001). A ROS-sensitive peroxidase (RIP1) is induced by NF treatment, suggesting that ROS levels are regulated by NFs (Cook et al., 1995; Ramu et al., 2002). ROS levels in *M. truncatula* roots start to decline 30–60 min after NF treatment (Lohar et al., 2007; Shaw and Long, 2003b). The response can be activated by 100 pM NF but is not activated by sulfated chitotetraose or non-sulfated NFs. The decline was absent in *nfp* mutants but was present in Sym pathway mutants, suggesting that it is involved in a separate signaling process. Exogenous ROS treatment prevented NF-induced root hair swelling and branching, suggesting that the ROS decline is involved in root hair deformation, perhaps by preventing the activation of plant defence responses (Lohar et al., 2007). It seems likely that the transient ROS increase and the later, more gradual decline in ROS may have separate signaling roles in nodulation.

54.11 CALCIUM SIGNALING IN ROOT HAIRS: LESSONS FROM APICAL GROWTH

Gradients of calcium, pH and ROS production are required for apical growth of root hairs (Herrmann and Felle, 1995; Jones et al., 1995; Foreman et al., 2003). These gradients oscillate with the same period but are out of phase with oscillations in the rate of cell expansion (Monshausen et al., 2007, 2008). Calcium ion influxes across the plasma membrane at the tip of cells are responsible for the calcium gradient (Herrmann and Felle, 1995; Very and Davies, 2000). The *A. thaliana rhd2* mutant lacks the tip-focused calcium gradient and tip ROS production and has stunted roots and short root hair cells (Wymer et al., 1997; Schiefelbein and Somerville, 1990). *RHD2* encodes a plasma membrane NADPH oxidase (AtRBOHC) and ROS treatment to *rhd2* roots raised cytoplasmic calcium levels in the root hairs and restored cell expansion but this was not limited to the tip, suggesting that the sub-cellular localization of ROS production is important for appropriate polar cell expansion. Plant NADPH oxidases or RBOHs (respiratory burst oxidative homologs) contain calcium-binding EF-hand motifs and calcium ions have been shown to regulate their activity *in vitro*, suggesting that a positive-feedback loop between calcium and ROS is involved in regulating apical growth (Sagi and Fluhr, 2001; Keller et al., 1998; Takeda et al., 2008). Elevation of the pH of the external medium surrounding *rhd2-1* mutants to 6.0 restored normal root growth along with the tip-focussed calcium gradient (Monshausen et al., 2007). However, the mutants had lower ROS levels in root hairs and lacked the oscillations in ROS production at the tip, suggesting that either pH lies downstream of ROS or that they may act together to regulate root hair growth.

The calcium gradients are thought to provide directional regulation of apical growth by regulating exocytosis and actin reorganization (Rato et al., 2004; He et al., 2006). Alongside any signaling roles, ROS and pH may also directly regulate cell wall structure. ROS are involved in peroxidative-cross linking of cell wall polysaccharides and pH is involved in cell expansion (Cosgrove, 1999; Bibikova et al., 1998; Kjellbom et al., 1997; Kerr and Fry, 2004). Artificially raising the pH of the external medium surrounding *A. thaliana* roots to 8.0 or application of ROS arrests root tip growth, whereas decreasing the external pH to 4.5 or treatment with the ROS scavenger MCLA (methoxylated *Cypridina luciferin* analog) causes root tip bursting, suggesting ROS and a rise in apoplastic pH promote cell wall hardening during the slower growth phases.

Root hair apical growth is co-ordinated by ROP GTPases, which associate with the plasma membrane at the apex of the cell. There are 11 members of the ROP GTPase family in *A. thaliana* and they are involved in processes including abscisic acid (ABA) responses, pollen tube growth, cell shape formation and low oxygen response (Craddock et al., 2012). ROP GTPases switch between an activate state when they are bound to guanosine triphosphate (GTP) and an inactive state when they are bound to guanosine diphosphate (GDP). ROPs are regulated by RopGEFs, which exchange the GDP for GTP to return the ROP to its active state; RopGAPs, which promote the intrinsic GTPase activity of the ROP to inactivate it; and RhoGDIs, which sequester ROPs in the cytosol to prevent them being activated by the RopGEFs (Berken and Wittinghofer, 2008; Bos et al., 2007; Carol et al., 2005; Kost, 2008). The *A. thaliana scn1* mutant is defective in a RhoGDI and develops multiple root hair initials (Carol et al., 2005). *AtROP2* is mislocalized in this mutant demonstrating the importance

of the regulation of ROP GTPase subcellular localization for regulation of polar growth. In *A. thaliana* ROP GTPase genes can have redundant and non-redundant functions and *AtROP2*, *AtROP4*, and *AtROP6* are involved in root hair apical growth (Jones et al., 2002, 2007; Molendijk et al., 2001; Yang et al., 2007; Duan et al., 2010). *AtROP2* and *AtSCN1* (a RhoGDI) are required for AtRBOHC-mediated ROS production during root hair growth (Carol et al., 2005; Jones et al., 2007).

Regulation of polar growth is essential during rhizobial infection to mediate root hair curling around attached bacteria and the development of the infection thread. It seems likely that the mechanisms for regulating the processes for root hair growth may have been co-opted for rhizobial infection. NF treatment induces root hair elongation, but unlike apical growth this is independent of ethylene indicating that if NF can activate the apical growth machinery it does so downstream of the ethylene receptors (Oldroyd et al., 2001b). Spot inoculation of NF to the surface of *M. truncatula* root hairs is sufficient to alter the axis of polar growth to generate root hair curling (Esseling et al., 2003). Recently, evidence of the involvement of ROP GTPase signaling in nodulation has been emerging. Three *M. truncatula* ROP GTPase genes are up-regulated in roots and root hairs after inoculation with *S. melliloti* (Liu et al., 2010). RNAi knockdown of *MtROP9* inhibits an NF-induced ROS response and results in the formation of fewer nodules (Kiirika et al., 2012). In *Lotus japonicus LjROP6* is up-regulated after inoculation with *M. loti* and the protein interacts with the NF receptor NFR5 *in planta* (Ke et al., 2012) (Fig. 54.4). *LjROP6* RNAi lines showed an inhibition of infection thread growth through the cortex suggesting that ROP6 may promote infection thread development from the epidermis into the cortex, perhaps through regulation of the cytoskeleton (Ke et al., 2012). The RNAi lines also produced fewer nodules and the early nodulation genes *NIN* and *ENOD40* are down-regulated compared to wildtype after inoculation with *S. meliloti.* These results indicate that either *LjROP6* may be involved in the Sym pathway or it may lie in a parallel pathway, perhaps primarily involved in regulating infection but with a role in the positive feedback of the Sym pathway as part of the co-ordination of nodule development and bacterial infection.

Part of the function of ROP GTPases in nodulation may involve the regulation of NADPH-dependent ROS production. The *MtRbohA* gene is up-regulated in nodules during the *M.truncatula-S. melliloti* Sym and expression of *MtRbohA* appears to be restricted to the nitrogen-fixing zone of the nodules (Marino et al., 2011). Plants inoculated with *S. meliloti* mutant strains *nifH* and *bacA*, which are unable to form functioning nodules, had lower *MtRbohA* expression and *MtRbohA* RNAi lines had lower nitrogen fixation activity in nodules (Marino et al., 2011). Coinciding with the decline in ROS after NF treatment, *MtRBOH2* and *MtRBOH3* are transiently down-regulated at 1 h in an NFP-dependent manner in *M. truncatula* (Lohar et al., 2007). Another member, *PvRbohB* is expressed in several tissues including developing nodules and in infected root hairs in *P. vulgaris* (Montiel et al., 2012). RNAi lines have reduced ROS production, fewer nodules and the infection threads are impaired in progression from the epidermal cells into the cortex. The few nodules that form in the *PvRbohB* RNAi lines have infection threads that are wider and more irregular in shape, indicating that *PvRbohB* is involved in infection thread development. It would be very interesting to know if any of

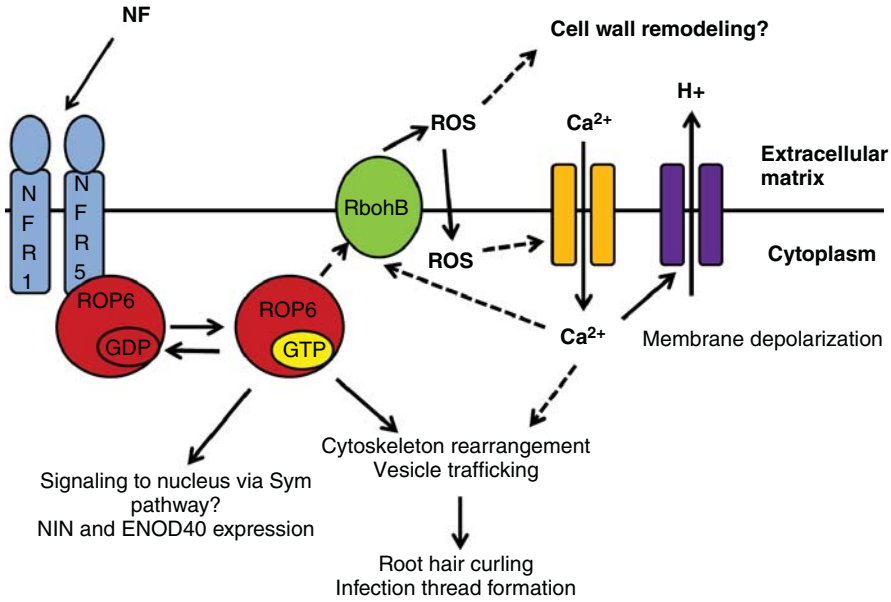

Figure 54.4 Initiation of the NF-induced calcium influx. ROP6 signaling is activated by NF binding to ROP6-interacting partner NFR5. ROP6 activates Rboh activity producing ROS, promoting cell wall remodeling and activating a calcium influx. Calcium ions and ROP GTPases regulate cytoskeleton rearrangement and vesicle trafficking to generate root hair curling around attached rhizobia and regulate infection thread formation. ROP6 also induces *NIN* and *ENOD40* gene expression, perhaps via the Sym pathway. Cycling of ROP GTPases between GDP and GTP bound states by RopGEFs and RopGAPS (not shown) and sequestering in the cytoplasm by RhoGDIs ensure ROP activity can be appropriately localized to drive new membrane formation in the appropriate location and direction.

the ROP GTPases or Rbohs that are involved in bacterial infection are required for the induction of the calcium influx.

In summary, drawing on parallels between rhizobial infection in legumes and polar root hair growth in *A. thaliana* it seems likely that NF induction of the calcium influx is mediated by ROP GTPase signaling (Fig. 54.4). At the root hair tip, perception of NFs by the NF receptors NFR5/NFR1 leads to the activation of ROP6, which could promote Rboh-dependent ROS production. The ROS production may promote cell wall remodeling and/or the activation of the calcium influx and membrane depolarization. In *A. thaliana* ROP GTPases are master regulators of polar growth, so in legumes the calcium influx may be involved in ROP GTPase regulation of cytoskeletal rearrangement and vesicle trafficking leading to root hair curling and infection thread formation.

54.12 CONCLUDING REMARKS

The NF-induced calcium spiking and calcium influx responses are spatially and genetically separable. Mathematical modeling is providing insight as to how calcium spiking can be generated by ion fluxes across the nuclear membranes and detailed studies of CCaMK are gradually unraveling the regulation of this unique protein. However, there are still several major gaps in our understanding of the generation of calcium spikes and how they are decoded to activate nodulation gene expression. Identification of the calcium channel responsible for calcium release from the nuclear lumen would be a major advance and may also provide insight as to how the NF signal is relayed by secondary messengers from the plasma membrane to the nucleus to activate calcium spiking. An outstanding question is whether the calcium spiking signal itself encodes rhizobial or mycorrhizal-specific information, or is a more general Sym signal with downstream Sym pathway signaling modulated by parallel pathways that determines specificity. As CCaMK seems to be at the point of divergence of the two pathways it is likely that understanding how it is regulated will provide further insight into how the specificity is achieved.

What role the NF-induced calcium influx plays in nodulation is still a mystery. Analogy with apical growth of root hairs suggest that the calcium influx may be interacting with spatially and temporally co-incident NF-induced ROS and pH changes to signal alterations in polar growth of the root hair required to generate root hair curling and infection thread formation. Consistent with this several ROP GTPase and NADPH oxidases involved in bacterial infection have been identified, and in the future it will be interesting to find out whether they are required to generate the calcium influx. If any ROP GTPase or NADPH oxidases are required for the activation of the calcium flux it would provide a missing link between the perception of NF and the calcium influx, and strengthen evidence for a role of the calcium influx in bacterial infection.

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Chapter 55

Signaling and Communication between Actinorhizal Plants and *Frankia* during the Intracellular Symbiotic Process

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55.1 INTRODUCTION

Actinorhizal root nodules are specialized organs that result from the interaction between the gram-positive nitrogen-fixing actinomycete *Frankia* and roots of dicotyledonous plants collectively called actinorhizal plants. Inside the infected cells of the nodule, *Frankia* reduces atmospheric nitrogen to ammonium for the benefit of the host plant that in return provides reduced carbon to the microsymbiont (Chaia et al., 2010; Franche and Bogusz, 2012; Pawlowski and Demchenko, 2012; see also Chapters 42, 43). The actinorhizal symbiosis is found among 25 genera of plants, mainly trees and shrubs, belonging to eight plant families in the orders Fagales (*Betulaceae*, *Casuarinaceae*, and *Myricaceae*), Rosales (*Rosaceae*, *Eleagnaceae*, and *Rhamnaceae*) and Cucurbitales (*Datiscaceae* and *Coriariaceae*). The symbiotic process occurs in conditions of nitrogen deficiency and results in high rates of nitrogen fixation comparable to those found in legumes (Diem and Dommergues, 1990). Besides their contribution to the global biological nitrogen fixation process, some actinorhizal species such as *Alnus glutinosa* and *Casuarina equisetifolia* have great economic value as timber and fuel wood, and also contribute to land reclamation and protection of coastal areas (Diouf et al., 2008; Dawson, 2008; Zhong et al., 2010).

Actinorhizal nodulation can occur by an intercellular penetration mechanism in the orders Rosales and Cucurbitales (Miller and Baker, 1985) or by intracellular penetration via root hair infection in the order Fagales (Callaham et al., 1979). It should be noted that a single *Frankia* strain can exhibit different infection mechanisms, whereas the host plant has the capability for only one mode of infection (Miller and Baker, 1986). First identified in *Eleagnaceae* (Miller and Baker, 1985), the intercellular infection process does not involve the infection of root hairs. Current knowledge on this infection process is described in the chapter dedicated to *Discaria trinervis* (Chapter 43), while this chapter will focus on the molecular mechanisms underlying intracellular infection by *Frankia*.

Four major stages can be distinguished during the intracellular infection process: the presymbiotic stage that involves perception and recognition of specific and still unknown plant and *Frankia* signal molecules, the infection of root hairs by *Frankia* hyphae, the induction and infection of a prenodule resulting from the division of root cortical cells, and the induction and invasion of a nodule primordium initiated from divisions in the root pericycle (Berry and Sunnel, 1990; Wall and Berry, 2008; Santi et al., 2013). The resulting actinorhizal nodule is a coralloid cluster of small lobes; each of them possess a central vascular system and *Frankia* infected cells (fic) in the expanded cortex. Behind the nodule meristem, there is a progression in the stage of infection, with the youngest stages being closest to the apex.

The development of the actinorhizal symbiosis is associated with major changes in *Frankia* (Alloisio et al., 2010; Beauchemin et al., 2012) and plant gene expression (Hocher et al., 2006, 2011; see Chapter 42). Recent detailed cytological analyses in RNAi plants as well as the

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characterization of promoters from symbiotic genes, have proved valuable in revealing the function of genes involved in the symbiotic process with the actinomycete (Svistoonoff et al., 2010a; Franche and Bogusz, 2012; see Chapter 43). This chapter will report on the recent advances in deciphering the molecular dialog during the intracellular infection process, with an emphasis on the data obtained with the tropical tree *Casuarina glauca* where genetic transformation tools are available (Franche et al., 1997, Smouni et al., 2002), together with transcriptomic resources (Hocher et al., 2011; Tromas et al., 2012).

55.2 THE PRESYMBIOTIC DIALOG

55.2.1 Host-*Frankia* Specificity

Successful actinobacterial symbiosis requires a compatibility between the host plant and its symbiont. Phylogenetic analyses reveal that *Frankiae* form a coherent clade within the actinobacteria, and that strains generally fall into three major groups or clusters (Normand et al., 1996, Benson and Clawson, 2000; Hahn, 2008). Cluster I consists of strains isolated from plants belonging to the order Fagales; they have the most specific host range and are only able to interact with plants belonging to this clade. A subgroup of cluster I infects the family *Casuarinaceae*, and appears to have evolved even higher levels of specificity, as some *Frankia* are only able to nodulate *Casuarina* and *Allocasuarina* in natural conditions. Strains belonging to cluster III have a wider host range and can interact with plants belonging to five families within the two distant plant orders Rosales and Fagales. No strain belonging to cluster II has yet been cultured (see Chapter 43).

The molecular basis for host specifity of interaction between *Frankia* and the plant hosts remains unknown due to the difficulty to develop genetic tools for the actinomycete.

55.2.2 *Frankia* Signals

The initiation of the actinorhizal symbiotic relationship involves a molecular dialog between the plant and its bacterial partner. Bacteria-free filtrates from exponentially growing cultures of *Frankia* contain a factor(s) that induces root hair deformation (RHD) in the host (Fig. 55.1), thus indicating the presence of specific diffusible molecule(s) (see also Chapter 43). The susceptible root zone remains confined to a region of the root just behind the root tip, from the location of young emerging root hairs to the location of mature root hairs.

The chemical structure of *Frankia* factors involved in the symbiotic process remains unknown so far. However, some biochemical properties have been established for the strain ACoN24d nodulating *A. glutinosa*. The RHD factor(s) has a molecular weight of about 5 kDa and, whereas in

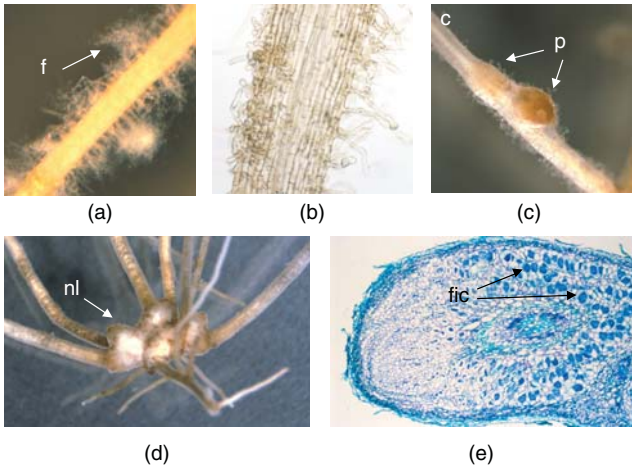

Figure 55.1 Nodulation of *Casuarina glauca* by the actinomycete *Frankia*. (a) Chemoattraction of *Frankia* (f) hyphae observed 24 h after inoculation of *C. glauca* plants deprived of nitrogen. (b) Root hair curling on lateral roots of *C. glauca*. (c) Prenodules (p) resulting from root hair infection. (d) Two-month-old nodule of *C. glauca* exhibiting several nodular lobes (nl). (e) Longitudinal section of an actinorhizal nodular lobe with *Frankia*-infected cells (fic).

legumes Nod factors are amphiphilic and chitinase sensitive (Dénarié et al., 1996), *Frankia* factors from the symbiotic strain ACoN24d of *Alnus* were found to be hydrophilic and resistant to endochitinase and exochitinase from *Streptomyces griseus*. However, *N*-acetyl-glucosamine, the subunit of the Nod factor backbone, has been detected in the *Alnus* root hair deforming fraction, leaving open the existence of a Nod-factor-related *Frankia* compound (Cérémonie et al., 1999). The recent identification of *C. glauca* promoters from symbiotic genes that are specifically induced by the supernatant of *Frankia* CcI3 provides a valuable tool for the future purification of the corresponding molecules (Rhizogenesis, Montpellier, France).

55.2.3 Plant Signals

Plants have recruited phenolics as signal molecules to facilitate interactions with other organisms (Weston and Mathesius, 2013; see Chapters 42, 50). In Legumes, the host plant secretes phenolic compounds, essentially flavonoids, that attract symbiotic rhizobia and further activate the transcription of the *nod* genes that are essential for both host range and nodulation (Wasson et al., 2006; Zhang et al., 2009). Several studies have shown that flavonoid-like compounds also contribute to influence nodulation of actinorhizal plants by *Frankia* (Abdel-Lateif et al., 2012). In *Alnus* sp., nodulation was enhanced by the addition of flavonones (Benoit and Berry, 1997) or flavonols compounds (Hughes et al., 1999). A general reorganization of protein biosynthesis was also observed when three *Frankia* strains, ACN14a, M16467, and Ea112, were treated with seed

phenolic extracts from the actinorhizal plant *Myrica gale* (Bagnarol et al., 2007). Additional studies in *Myricaceae* suggested that the interaction between root flavonoids and actinobacteria was strain specific (Popovici et al., 2010).

In *C. glauca*, several genes involved in the flavonoid pathway have been identified and shown up-regulated following plant inoculation by *Frankia* Cci3 (Auguy et al., 2011; Hocher et al., 2011). Transcript analysis suggested an increase in expression of the chalcone synthase isomerase and the isoflavone reductase genes after inoculation by *Frankia*, whereas isoflavone reductase and flavonoid-3′,5′-hydroxylase transcripts accumulated in mature nodules. To further investigate the role of flavonoids during actinorhizal symbiosis, the chalcone synthase gene *CgCHS1* (Laplaze et al., 1999) was down-regulated in *C. glauca* using an RNA interference approach. This enzyme was chosen as a target as it is the first enzyme of the flavonoid pathway, catalyzing the synthesis of naringenin chalcone and naringenin flavanon, from which diverse flavonoids are derived. Data obtained in RNAi-*CgCHS1* roots showed that knockdown of chalcone synthase expression reduced the level of specific flavonoids and resulted in severely impaired nodulation (see also Chapter 42). A 1-month delay in nodulation was observed in RNAi plants and 12 weeks after inoculation, 56% of control plants were nodulated, while only 30% of *CgCHS1*-silenced roots contained nodules; furthermore, the number of nodules per RNAi-*CgCHS1* plant was reduced by about twofold. Nodule formation was rescued by supplementing the RNAi-*CgCHS1* plants with naringenin, which is an upstream intermediate in flavonoid biosynthesis. These data resulting from the functional analysis of *CgCHS1* in the actinorhizal tree *C. glauca* provide a direct evidence for the role of flavonoids during the infection process by *Frankia*.

55.3 PLANT RESPONSE TO *FRANKIA* IN THE INTRACELLULAR PATHWAY

55.3.1 Root Hair Deformation (RHD) and Infection

The earliest visible event in intracellular infection process is the infection of root hairs by *Frankia* hyphae (Callaham et al., 1979; Prin and Rougier, 1987). Epidermal cells located in the zone of root hair growth constitute a privileged site of interaction with the actinomycete. Within 8–24 h, hyphae are attracted by root hairs and, following their colonization by *Frankia*, root hair branching or curling is observed (Miller and Baker, 1986; Berry and Sunnel, 1990) (Fig. 55.1a,b). Some hyphae captured within a curled root hair partly dissolve the plant cell wall and enter the root hair by invagination of the plant plasma membrane. When the bacterium penetrates the cell wall, it becomes an intracellular endophyte. The host deposits new cell wall material at the site of infection, forming a so-called infection thread that is able to grow toward the base of the trichoblast. Infection threads penetrate further into cortical cells by local degradation of the cell wall.

55.3.2 Prenodule and Nodular Primordium

Besides RHD, some responses are also observed in root cortical cells and pericycle cells (Callaham and Torrey, 1977). Some cell divisions are observed in the root cortex, close to the *Frankia* infected root hair, leading to the development of a small protuberance called the prenodule (Fig. 55.1c; see also Chapter 42). Hyphae encapsulated in the infection threads progress toward the mitotically active zone and then further invade some cells of the prenodule. *Frankia* proliferates in the infected prenodule cells that undergo considerable hypertrophy. When multiple root hair infection occur, several prenodules can be observed on a same root (Fig. 55.1c). The prenodule stage is neither observed during the intercellular actinorhizal infection process nor the intracellular symbiotic process between legumes and rhizobia. Once the prenodule is established, one or more primordium are initiated from the pericycle. As the prenodule stage appears as an obligatory step for nodule organogenesis in the intracellular infection process, it is likely that specific signals are necessary to induce cell divisions in the pericycle cells.

Although the function of the prenodule remains to be elucidated, *in situ* hybridizations experiments together with studies of transcriptional fusions between promoters from symbiotic genes and reporter genes have provided some preliminary information concerning gene expression in the prenodule (Laplaze et al., 2000a). In *C. glauca*, fic were found to express the *nifH* gene encoding the iron protein subunit of the nitrogenase complex, and the symbiotic hemoglobin gene, thus suggesting that *Frankia* hyphae in hypertrophied cells of the prenodule can fix nitrogen. These data are further confirmed by the differentiation of nitrogen-fixing vesicles in the prenodules of *A. glutinosa* (Berry and Sunnel, 1990). So far, actinorhizal promoters conferring gene expression in nodule-infected cells exhibit a similar pattern of expression in prenodule-infected cells. In *Casuarinaceae* plants genetically transformed with a ß-glucuronidase (GUS) gene driven by the promoters from the genes *Cg12* and *CgAUX1*, encoding an infection-associated serine protease (Svistoonoff et al., 2004) and an auxin influx carrier protein (Péret et al., 2007) from *C. glauca*, respectively, GUS activity was observed in both prenodule and nodule cells infected by the actinobacteria. Interestingly, similar data were observed with the promoter from the early symbiotic gene *MtEnod11* from *Medicago truncatula* encoding a putative cell wall

repetitive proline-rich protein (Svistoonoff et al., 2010b), and more recently, with the promoter from *MtDMI2* coding a leucine-rich-repeat receptor kinase (Bersoult et al., 2005) involved in the Nod-factor signaling cascade (Rhizogenesis, IRD Montpellier, France). All these data lead to the hypothesis that the prenodule could be a primitive form of a nitrogen-fixing symbiotic organ. In the future, further transcriptome analyses targeted to the prenodule tissue will help clarify the role of this obligatory developmental step in the actinorhizal infection process.

55.3.3 Actinorhizal Nodule Development

The nodular primordium arise from divisions in pericycle cells opposite to a protoxylem pole and close to the prenodule (Berry and Sunnel, 1990). This nodule primordium will grow and become infected by *Frankia* that advances by cell wall penetration through the prenodule and the nodule protoperiderm, toward the nodule meristem. After penetration into cortical cells, the endophytic hyphae proliferate, leading to the enlargment of the infected cells. When the infected cell matures, the tips of the hyphae eventually differentiate into vesicles that will fix nitrogen (Newcomb and Wood, 1987). The mature actinorhizal nodule consists of multiple lobes, each of which is a modified lateral root with a central vascular bundle (Fig. 55.1d,e).

Within the cortex, infected cells occur in layers surrounded by smaller non-infected cell layers that contain heavy deposits of phenolic compounds. It was believed that the high amounts of phenolics in uninfected cells may represent a defense response to *Frankia* infection and could contribute to restrict the endophyte to certain regions in the cortex, preventing its spread in the vascular tissue, periderm and meristem. However, recent data arising from the histological analysis of the RNAi-*CgCHS1* nodules from *C. glauca* are not in favor of this hypothesis. Although the amount of phenolic compounds was drastically reduced in uninfected cells of the RNAi nodules, fic still occurred in layers surrounded by uninfected cells and the compartmentation of the cortex of the nodule lobe was conserved.

55.4 PLANT GENES EXPRESSED IN *FRANKIA*-INFECTED CELLS

55.4.1 Functional Analysis of Genes from the Common Symbiotic Pathway

The understanding of the molecular mechanisms involved in the actinorhizal symbiosis has benefited from the recent breakthroughs obtained on the model Legumes *Medicago* and *Lotus*. Symbiotic legume mutants unable to form nodules have contributed to the identification of genes controlling early stages of the perception and signal transduction pathway of the Nod factors (Madsen et al., 2010; Oldroyd et al., 2011; see Chapters 59, 63). Their analysis revealed that some of them were also affected in the arbuscular mycorrhizae (AM) symbiosis, thus indicating that the two symbiotic interactions overlap in the early steps of the symbiotic interactions. Among this shared "common SYM pathway," (also known as CSSP; see Chapters 42, 54, 110) the homologs of the symbiotic genes *DMI2/SymRK* and *DMI3*/*CCaMK* from legumes have been characterized in *C. glauca* using an RNAi approach and the spatiotemporal analysis conferred by the promoter region.

In Legumes, the receptor kinase DMI2/SymRK encodes a leucine-rich-repeat receptor kinase that is highly expressed during the infection process by Rhizobium and also required for endomycorrhization (Endre et al., 2002; Stracke et al., 2002). Downregulation of *CgSymRK* resulting from an RNA interference approach revealed that the frequency of nodulated RNAi-*CgSymRK* plants was reduced twofold compared to *C. glauca* control plants. In addition, a range of morphological alterations was observed in the down-regulated *CgSymRK*-nodules (Gherbi et al., 2008; Benabdoun et al., 2011). Additional experiments revealed that CgSymRK was also necessary for the establishment of the symbiosis with the arbuscular mycorrhiza *Glomus intraradices*. The knockdown of *CgSymRK* was seen to strongly affect penetration of the fungal hyphae into the root cortex, thus revealing the key role of CgSymRK in root endosymbioses in *Casuarina*, and the conservation of SymRK function between legumes and actinorhizal plants (Gherbi et al., 2008). Similar results were obtained following the functional analysis of *DtSymRK*, a *SYMRK* sequence isolated from the actinorhizal plant *Datisca glomerata* (Markmann et al., 2008). The most recently targeted gene is *CgCCaMK* that encodes a Calcium and Calmodulin-dependant kinase homolog to the *DMI3*/*CCaMK* gene from legumes (Lévy et al., 2004; Mitra et al., 2004; see Chapter 54). Functional analysis of the RNAi plants also indicate that this gene is necessary for appropriate root infection by *Frankia* and nodule development in *C. glauca*, thus suggesting that the entire common SYM pathway could be conserved in actinorhizal plants (see also Chapters 59, 110).

55.4.2 Other Genes Expressed during the Infection Process

cg12 was one of the first actinorhizal symbiotic gene isolated from *C. glauca* (Laplaze et al., 2000b). It encodes a subtilisin-like serine protease (subtilases). Subtilases are a super-family of proteases and are thought to play a role in several different aspects of plant development, response to pathogens and lateral root development (Schaller et al.,

2012). The regulation of *cg12* expression and its possible role during actinorhizal nodule infection was investigated following the analysis of transgenic *cg12-gus* and *cg12-gfp* transgenic plants of *C. glauca*. Expression of the reporter genes was observed when *Frankia* hyphae invaded deformed root hairs and in root prenodule and nodule cortical cells containing growing infection threads (Svistoonoff et al., 2004). The correlation of *cg12* expression with plant cell invasion lead to suggest that CG12 could participate in cell wall weakening during *Frankia* infection; it might also be involved in the maturation of a polypeptide as part of a signaling cascade in response to infection.

A clone for type I metallothionein (MT) (*cgMT1*) was then isolated from a *C. glauca* nodule cDNA library (Laplaze et al., 2002). MTs are defined as low molecular weight cysteine-rich proteins that can bind heavy metals and may play a role in their intracellular sequestration and transportation. They are also involved in response to stresses like wounding, pathogen infection and leaf senescence and function as antioxidants (Hassinen et al., 2011). In transgenic *Casuarinaceae* plants, the *cgMT1* promoter was shown to be primarily active in large fic of the nodule nitrogen-fixing zone, roots and the oldest parts of the shoots. Induction experiments revealed that the promoter P*cgMT1* responded to wounding, oxidative stress and pathogen infection, thus suggesting that *cgMT1* could be involved in antioxidant defence against reactive oxygen species induced during the symbiotic process (Obertello et al., 2007).

In 2007, the auxin influx carrier *CgAUX1* was studied. Using the *GUS* gene driven by the *CgAUX1* promoter, it was clearly shown that auxin plays an important role during plant cell infection in actinorhizal symbiosis (Péret et al., 2007; see also Chapter 47). *CgAUX1-GUS* was found to be strongly expressed both during root hair infection and cortical cell invasion by *Frankia*. It was also reported that *CgAUX1-GUS* was highly expressed in the root primordium, whereas no reporter gene activity was observed in the nodule primordium. This result further indicates that, although the actinorhizal nodule is comparable with a symbiotic lateral root, the molecular mechanisms involved in primordia initiation in lateral roots may differ from those in actinorhizal nodules.

55.5 CONCLUSIONS

Progress in the knowledge of the actinorhizal symbiosis has been hampered by the difficulties to develop functional analysis of candidate symbiotic genes in *Frankia*, together with the lack of green fluorescent protein (GFP) tagged *Frankia* strains to easily follow the infection process in wildtype and RNAi actinorhizal plants. The complete genome sequences that are currently available for various *Frankia* strains, combined with the data arising from transcriptomic and proteomic analyses, provide a vast amount of information (Alloisio et al., 2007, 2010; Normand et al., 2007a, 2007b; Benson et al., 2011). Genes that are induced or repressed during the different stages of the symbiotic process can now be identified, while new attempts to develop targeted mutagenesis in *Frankia* are still in progress.

The sucessful functional analysis of symbiotic genes in the actinorhizal plants *C. glauca* and *D. glomerata* has revealed a common symbiotic signaling pathway in actinorhizal, legume and arbuscular mycorrhization (Gherbi et al., 2008, Markmann et al., 2008). There are still indeed many mechanisms unknown in the plant–*Frankia* interactions. In the next years, challenging aspects of actinorhizal research will be the identification of the signaling molecules involved in the control of host specificity, infection and nodulation, as well as the mechanisms by which these factors are perceived and transduced in the host plant. The understanding of the signals and genes that enable an actinorhizal plant to enter into symbiotic association with *Frankia* may help in the future to extend the ability to fix nitrogen outside the legume and actinorhizal plant families (see Chapter 108).

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Section 10

Infection and Nodule Ontogeny

Chapter 56

The Role of Hormones in Rhizobial Infection

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56.1 INTRODUCTION

Nodulation involves the local perception of a rhizobial signaling molecule called Nod factor (NF, see Chapter 51). These lipochitooligosaccharides induce two principal developmental outcomes, infection thread formation by rhizobia and nodule organogenesis, which depend on changes in the production, transport, and sensing of plant hormones (reviewed in (Oldroyd et al., 2011; Guinel and Geil, 2002)). Hormones are defined as regulatory substances produced in a cell and transported externally to elicit responses in specific cells or tissues. In plants this includes auxin, gibberellic acid (GA), jasmonic acid (JA) and, more recently, strigolactones (SLs). Nodulation is a multi-stage developmental process, and plant hormones are involved at every stage, from influencing the initial NF production by rhizobia to the formation of nodules. During nodulation, rhizobia typically attach near the tip of a growing root hair, which curls around the attached rhizobia to entrap them, creating the so-called infection pocket. The infection thread initiates from this infection pocket as a narrow tube around 1 μM in diameter. The infection thread then grows through the root hair cell as it becomes colonized by the rhizobia. The infection thread subsequently grows into the subtended outer cortical cells and finally into the cells of the growing nodule primordium, where it branches and eventually releases bacteria enclosed by the host membrane into nodule cells where they fix nitrogen. Although considerable effort has been directed at determining the role of hormones in nodulation, much of this work has been focused on nodule organogenesis, and, consequently, relatively little is known about hormonal involvement in rhizobial infection. Here, we review the role of SLs, JA, auxin, and GA in this process and consider their potential interactions.

56.2 STRIGOLACTONES ARE PHYTOHORMONES AND PLANT-MICROBE SIGNALS

SLs are carotenoid-derived terpenoids that act as endogenous plant hormones and as secreted signals to other organisms in the rhizosphere. Originally, it was discovered as a seed germination stimulant of the parasitic plant witchweed (Cook et al., 1966), and later as a component of root exudate that can induce hyphal branching in arbuscular mycorrhizal fungi (Akiyama et al., 2005), a process required for normal colonization of the host (Gomez-Roldan et al., 2008). SLs were shown to inhibit shoot branching and have been classified as phytohormones in 2008 (Gomez-Roldan et al., 2008; Umehara et al., 2008). Since then, SLs have been associated with a growing array of endogenous functions, influencing both shoot and root architecture (Ruyter-Spira et al., 2013). While the role of SLs in the arbuscular mycorrhizal symbiosis continues to expand (Yoshida et al., 2012), a new role in the legume-rhizobia symbiosis is beginning to emerge (Soto et al., 2010; Foo and Davies, 2011; et al., 2013).

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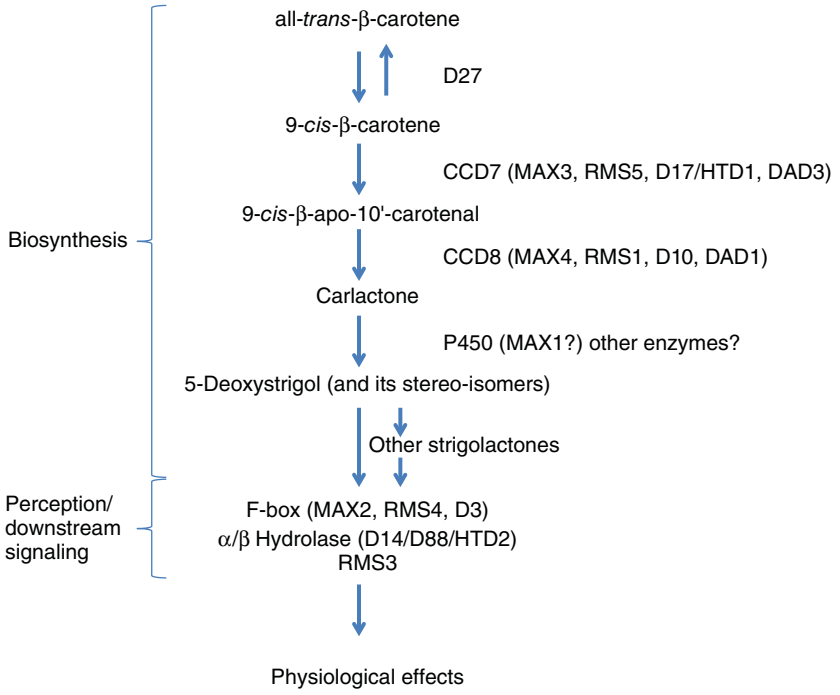

Figure 56.1 Strigolactone biosynthesis and downstream signaling. (*Source:* Figure adapted from (Ruyter-Spira et al., 2013.))

Functional diversity is accompanied by an expanding catalog of SL compounds, which now includes at least 15 members (Ruyter-Spira et al., 2013). SLs are synthesized from β-carotene using at least four biosynthetic steps, as shown in Figure 56.1 (Alder et al., 2012).

The sequential action of a β-carotene isomerase (DWARF27; D27) and two carotenoid cleavage dioxygenases (CCD7 and CCD8) produce a compound called carlactone that is able to stimulate seed germination in parasitic plants (Alder *et al*., 2012). At least one further step, perhaps involving a cytochrome P450 (MAX1) (Booker et al., 2005), is required to produce 5-deoxystrigol, an SL and likely precursor of other SLs (Ruyter-Spira et al., 2013). Additionally, several other genes have been implicated in SL perception or signaling (Fig. 56.1) including an F-box protein (Johnson et al., 2006; Stirnberg et al., 2007; Yoshida et al., 2012) and an α/β hydrolase (Arite et al., 2009; Hamiaux et al., 2012; Waters et al., 2012).

Much of our knowledge of SL biosynthesis and downstream signaling comes from mutants that display increased branching of the shoot. Cloning such mutants in rice (*dwarf*, *d*); pea (*ramosus*, *rms*); petunia (*decreased apical dominance*, *dad*) and *Arabidopsis* (*more axillary growth*, *max*) have revealed components of both SL biosynthesis and perception (Table 56.1). Reciprocal BLAST searches of the *Medicago truncatula* genome have revealed homologs of all previously characterized SL biosynthetic and perception/signaling genes (Liu et al., 2011; Challis et al., 2013), (Table 56.1).

56.3 STRIGOLACTONES IN NODULATION

The first evidence of a role for SLs in nodulation came from alfalfa (Soto et al., 2010). Treatment of *Sinorhizobium meliloti* inoculated plants with the synthetic SL GR24, stimulated a 30–40% increase in nodule number compared to untreated controls (Soto et al., 2010). In pea, the SL deficient *rms1* (orthologous to *ccd8*) displayed a significant reduction (approximately 40%) in nodule number, which could be rescued by GR24 treatment (Foo and Davies, 2011). Although the phenotype was reported for only a single allele of *rms1*, a significant decrease in nodule number was observed for another SL biosynthesis mutant, *rms5* (orthologous to *ccd7*) (Foo et al., 2013). SLs have also been shown to be required for normal numbers of nodules in *Lotus japonicus* where significantly fewer nodules were observed on a *Mesorhizobium loti* inoculated *ccd7*-silenced transgenic line (Liu et al., 2013). In contrast, nodule number was significantly increased (although nodules were significantly smaller than those on water-treated (WT) plants) in a pea SL-insensitive F-box (*rms4*) mutant (Foo et al., 2013). Notably, SL-insensitive mutants *d3* and *d14* have been shown to have increased SL production (Umehara et al., 2008; Arite et al., 2009), so one possible explanation is that the increased nodulation in the mutant is a consequence of higher SL levels. This appears consistent with a role of SLs in signaling to rhizobia in the rhizosphere, and suggests that endogenous perception of SLs is not required for infection

Table 56.1 Strigolactone genes and their *M. truncatula* homologs

Gene	Description	Characterized genes (Rice, Pea, Petunia, *Arabidopsis*)	*M. truncatula* homolog(s)*
D27	Carotene isomerase	*D27*, –, –, *AtD27*	Medtr1g471050†
CCD7	Carotenoid cleavage dioxygenase 7	*D17*, *RMS5*, *DAD3*, *MAX3*	Medtr7g045370
CCD8	Carotenoid cleavage dioxygenase 8	*D10*, *RMS1*, *DAD1*, *MAX4*	Medtr3g109610
MAX1	Cytochrome P450	–, –, –, *MAX1*	Medtr3g104560 Medtr1g015860
MAX2	F-box	*D3*, *RMS4*, –, *MAX2*	Medtr4g080020
D14	α-β hydrolase	*D14*, –, *DAD2*, *AtD14*	Medtr1g018320

*Established *M. truncatula* homolog or reciprocal BLASTP hit to Mt4.0v1 (http://blast.jcvi.org/Medicago-Blast/index.cgi) using characterized *Arabidopsis* protein sequence as the query.

†Described in Liu *et al*., 2011.

and initiation of nodule formation. However, treatment of *S. meliloti* with GR24 failed to induce a change in growth rate or induction of *nodC* (Soto et al., 2010).

Interestingly, the rice *d3* mutant shows strongly reduced mycorrhizal colonization while in *d14* mutants mycorrhizal colonization is increased (Yoshida et al., 2012). It seems clear from this that although D3 and D14 may be required for SL responses, they may also have additional roles. Along this vein, the D3 ortholog RMS4 has also been implicated in karrikin signaling (Nelson et al., 2011).

It is now clear that SLs influence nodulation. Whether the phytohormone acts as a signal to rhizobia, functions endogenously in the plant, or a combination of both, remains to be determined. If acting endogenously, it will be important to understand how SLs are perceived and how the downstream signaling pathway(s) influences nodulation. It will also be important to explore the potential crosstalk between SL signaling and other plant hormones. SLs have been shown to interact with auxin, ethylene, abscisic acid (ABA) and GA (reviewed in (Foo and Reid, 2012)) all of which have been associated with nodulation. Interestingly, their interaction with auxin and ethylene has been reported in the regulation of root hair elongation in *Arabidopsis* (Kapulnik et al., 2011). The positive influence of SLs on root hair growth was found to be dependent on MAX2 and to be mediated through ethylene. However, because NF-induced root hair growth is ethylene independent (Heidstra et al., 1997), and ethylene has strong repressive effects on infection and nodulation (reviewed in (Murray 2011)), it seems extremely unlikely that SLs are involved in the root hair growth that occurs during rhizobial infection. On the other hand, it has been shown that GR24 increases the GA/ABA ratio in *Arabidopsis* and witchweed seeds (Toh et al., 2012). Because GA has a positive role in nodulation (discussed below) and ABA inhibits nodulation (Suzuki et al., 2004) this seems a promising lead to understand the role of SLs in nodulation.

In summary, it is still uncertain whether SLs serve as an external or internal signal or both to enhance nodulation. SL biosynthetic and signaling mutants in model legume species are needed to help elucidate the role of this hormone.

56.4 JASMONIC ACID ACTS AS A PLANT HORMONE AND A LONG DISTANCE SIGNAL FOR RHIZOBIA

JA is a plant hormone that is widely known for its roles in plant defense to both necrotrophic pathogens and herbivores. JA is synthesized through what is commonly referred to as the octadecanoid pathway due to its 18 carbon precursor, linolenic acid. It is also implicated in other processes such as abiotic stress responses, pollen development, vegetative growth and senescence. Studies have shown that JA also has an important function in legume nodulation. It has a role in the dialogue that initially occurs between rhizobia and the host plant, and may also have a role in nodule development in the context of autoregulation of nodulation (AON).

56.5 A ROLE IN SIGNALING TO RHIZOBIA

The most recognized and studied plant-rhizobia signaling compounds are flavonoids that legumes exude from the root into the rhizosphere stimulating the expression of *nodD* genes in soil-borne rhizobia (see Chapter 50). This results in the expression of other *nod* genes and subsequently to NF production (Mulligan and Long, 1985). However, perhaps less well recognized is that JA and methyl jasmonate (MeJA) can also induce *nod* genes in *Rhizobium leguminosarum* (Rosas et al., 1998). Genistein is the main flavonoid secreted by soybean roots which is a powerful inducer of *nod* genes in *Bradyrhizobium japonicum* (Kosslak et al., 1987). However, JA and MeJA are more potent *nod* gene inducers than genistein on soybean roots, and the NFs activated by them are just as effective. This was measured using high-performance

liquid chromatography to determine the lipochitooligosaccharide (LCO) profiles of bacterial cultures after treatment. Also, the cumulative effects of JA/MeJA and genistein produce more LCOs than either alone (Mabood et al., 2006). Remarkably, when pre-inoculating *B. japonicum* with JA or MeJA, it was found that soybean plants produce an increased number of nodules in a concentration-dependent manner (Mabood and Smith, 2005).

In addition to its role in the induction of *nod* gene expression, JA appears to also have a role in plant flavonoid biosynthesis. The *M. truncatula* flavone synthase gene (*MtFNSII*) is induced both by *S. meliloti* and exogenous JA application (Zhang et al., 2007). Notably, flavonoids also serve as signals to another important root symbiont, arbuscular mycorrhizal fungi (Nair et al., 1991; Steinkellner et al., 2007). It has been suggested that the NF response to JA may reflect a more ancient form of the symbiosis where rhizobia entered via cracks in legume roots (Sun et al., 2006). The close relationship of rhizobia to *Agrobacterium* (Sawada et al., 2003), which are attracted to plant wounds, adds weight to this idea.

56.6 JA INHIBITS NF-SIGNALING

Whilst JA has a positive role in the signal exchange between rhizobia and the plant host, it appears to actively inhibit the plant's ability to positively respond to the NFs. Calcium spiking in and around the nucleus of rhizobia infected cells, in response to NF, is a crucial step in early signaling events during nodulation (see Chapter 54). JA was found to affect the maintenance of spiking, the spike period, and the responsiveness to NF (Sun et al., 2006). Application of 100 μM JA after the start of NF-induced calcium spiking resulted in the rapid suppression of spiking which was subsequently recovered when the JA source was removed and replaced with more NF. Lower concentrations of JA (10–50 μM) did not block calcium spiking, but increased the period between the spikes. From this observation that JA can inhibit NF signaling, we would predict a corresponding reduction of expression of nodulation genes. Indeed, JA has been shown to inhibit the expression of the early marker genes of NF response, *RIP1* and *ENOD11* in plants inoculated with *S. meliloti* (Miwa et al., 2006; Sun et al., 2006; Zhang et al., 2012). Exogenous MeJA also negatively affects the early stages of rhizobial infection. At concentrations of 10^{-4} M, it greatly reduces root hair curling and infection thread formation. The gene encoding the nodulation transcription factor (TF) *Nodule Inception* (*NIN*) has reduced expression at this concentration, suggesting that the effects of MeJA are upstream of *NIN*, which is consistent with a defect in early NF-signaling. Indeed, since mutants with deficient calcium spiking still have some root hair responses, a role for JA at the level of NF perception is indicated.

Evidence suggests that some of the effects of JA on nodulation may be through the production of ethylene which is a strong inhibitor of infection and nodulation (the effects of ethylene on nodulation are reviewed by (Murray, 2011)). A concentration of 1 μM JA was found to strongly inhibit nodulation, but this reduction did not occur when the JA was applied in combination with the ethylene biosynthesis inhibitor L-α-(2-aminoethoxyvinyl)-glycine (AVG) or if the ethylene insensitive *sickle* (*skl*) mutant was used. However, a higher concentration (10 μM) of JA inhibited nodulation independent of ethylene. The higher concentration of JA was also found to reduce the percentage of cells that showed calcium spiking in response to NF (Sun et al., 2006).

56.7 JA HAS A ROLE IN THE AUTOREGULATION OF NODULATION

Nodule development places high demands on plant metabolism, making the control of nodulation events crucial for plant success. The plant host does this through the process of AON (Ferguson et al., 2010). Signals from the root travel to the shoot after rhizobial inoculation (Delves et al., 1986). This is perceived by a leucine rich repeat receptor-like kinase called Nodule Autoregulation Receptor Kinase (NARK) in soybean (Krusell et al., 2002), Hypernodulation Aberrant Root Formation 1 (HAR1) in *L. japonicus* (Nishimura et al., 2002), and Super Numeric Nodules (SUNN) in *M. truncatula* (Schnabel et al., 2005). The phenotypes of *Gmnark*, *Ljhar1* and *Mtsunn* mutants feature hypernodulation.

There are many putative JA-biosynthesis/responsive genes that are regulated by *GmNARK*. This includes an allene oxide cyclase, a lipoxygenase and an allene oxide synthase (Kinkema and Gresshoff, 2008). Although *Gmnark* mutants have high levels of endogenous JA (Seo et al., 2007) and show an increase in JA biosynthesis/responsive genes, when inoculated with rhizobia, the expression of these genes is reduced (Kinkema and Gresshoff, 2008). Also, treatment with a chemical JA inhibitor (n-propyl gallate) generates a significant reduction in nodulation in *Gmnark* mutants, but not in wildtype plants. These results indicate a positive role for JA in nodulation (Kinkema and Gresshoff, 2008). Contrasting results are provided by work using *L. japonicus* where exogenous JA inhibited nodulation and the *Ljhar1* mutant showed enhanced sensitivity compared to WT (Nakagawa and Kawaguchi, 2006).

Does JA promote or suppress nodulation? It is possible that exogenous JA application has a strong negative impact on NF signaling as discussed above, while endogenous production of JA does not, possibly due to distribution of the active hormone.

Evidence has emerged recently that further indicates a positive role for JA in nodulation. The gene *PhyB* is

important for detecting low red:far-red (R/FR) light, a condition generally unfavorable for nodulation. In *phyB* mutants, or in WT plants grown in these suboptimal conditions, nodule numbers are strongly reduced (Suzuki et al., 2011). Both were also accompanied by a decrease in JA-responsive genes. When treated with JA, all the plants showed increases in both infection thread formation and nodule formation.

Overall, work in this area suggests that JA promotes nodulation. Rhizosphere signaling to rhizobia may be directly affected by JA, or by JA-induced increases in flavonoids. Alternatively, the induction of flavonoids by JA may serve to alter polar auxin transport (PAT), which may in turn influence infection. In addition, high levels of JA may have local negative effects on nodulation signaling. Host mutants for JA perception will help clarify whether JA acts mainly through its effects on rhizobia or if it also affects the host plant directly.

56.8 AUXIN'S ROLE IN NODULATION

Went (1926) originally showed that asymmetric application of the plant hormone auxin on decapitated Avena coleoptile causes it to bend in proportion to the amount of auxin applied (Went, 1926). Using this principle, in 1936, Thimann showed that sections of young pea nodules, both, from the base and the meristematic apex, cause significant bending of the avena coleoptile. As the activity was not only ascribed to the nodule meristem but to the entire nodule cortex, he hypothesized that auxin in nodules was perhaps related to tissue infected with rhizobia (Thimann, 1936). Auxin was thus established to play a role in symbiosis (see also Chapter 47). However, it was not until 1989 that Hirsch et al. showed that nodule-like structures could be initiated in the absence of bacterial symbionts (Hirsch et al., 1989). Further evidence that changes in auxin transport were important for nodulation was provided by studies using the promoter of soybean *GH3*, an auxin responsive gene, fused to a β-glucuronidase (*GUS*) reporter. Using this tool, Mathesius et al. noted a striking but transient arrest in transport at the site of infection as early as 24 h post infection followed by a strong increase at the same position where the nodule later initiated (Mathesius et al., 1998; see Chapter 47). Remarkably, purified NFs were shown to disrupt auxin transport in a similar manner to naphthylphthalamic acid (NPA) (Boot et al., 1999; Pacios-Bras et al., 2003).

Directional or "PAT" refers to the cell to cell movement of auxin mediated by members of the AUX-LAX family of influx carriers and efflux transporters like the PIN-formed (PIN), P-glycoprotein (PGP) family proteins (Friml, 2003). The PGPs are members of the B subfamily of the ABC transporters. To date, four members of the family have been shown to transport auxin in *Arabidopsis* (Noh et al., 2001; Noh et al., 2003; Geisler et al., 2005; Terasaka et al., 2005; Kamimoto et al., 2012). In *M. truncatula*, there are ten *MtPIN* genes, five *AUX-LAX* genes (Schnabel and Frugoli, 2004) and based on the latest release of the *Medicago* genome Mt3.5, we have found at least 35 genes encoding full ABC subfamily B type transporters.

Auxin acts as a developmental trigger primarily by creating local cellular gradients in the existing auxin stream using this PAT machinery. Both in *Medicago* and *Lotus*, within the first 50 h post infection the soybean auxin marker *GH3* expression accumulates at the site of inoculation (Mathesius et al., 1998; Pacios-Bras et al., 2003; see Chapter 47). Upon infection, both the stably transformed *GH3* marker and the synthetic auxin marker DR5 in *Medicago* hairy roots (Huo et al., 2006) show an arrest in auxin transport at the inoculation site. In determinate nodules of stably transformed *DR5*:*GFP* (green fluorescence protein) lines of *Lotus*, 72 h post infection, marker activity is observed in a few cells just below the infected root hair cell. As nodule development progresses, the actively dividing cortical cells of the nodule maintain this *DR5* expression until the rhizobia enter the cortex (Suzaki et al., 2012). By 6 days post infection, *DR5* marker expression is restricted to the outer cortex of the nodule (Takanashi et al., 2011). The authors suggest that as the DR5 expression pattern seems to mimic meristem activity within the nodule, restriction of the marker expression to the periphery of a determinate nodule occurs because the parenchymatic cells in this region retain meristematic activity. In *Medicago*, DR5 expression in mature nodules is especially strong in the vascular bundles (Guan et al., 2013).

Plant flavonoids act as natural auxin transport inhibitors (ATIs) by competing with NPA binding sites (Jacobs and Rubery, 1988) and are also referred to as natural ATIs. Interestingly, a screen for NPA binding proteins, a synthetic ATI, identified members of the ABCB sub-family B type auxin efflux transporters (Noh et al., 2001; Murphy et al., 2002). Since the inhibition of auxin transport by NPA was shown to initiate nodule like organs (Hirsch et al., 1989), it follows that in the absence of endogenous ATIs, nodule number should be affected. Wasson et al. showed that in *M. truncatula*, by silencing the enzyme chalcone synthase which catalyses the first step of the flavonoid biosynthesis pathway, nodulation can be eliminated (Wasson et al., 2006). Conversely, in the hyper-nodulating mutant of *L. japonicus har1*, a stronger *DR5* expression was associated with a wider cortical area of expression and excessive cell division in comparison to the wild type (Suzaki et al., 2012). Auxin can thus be conclusively ascribed to have a positive role in nodule organogenesis. Moreover, observations that in the *M. truncatula* hypernodulating mutants *sickle* and *sunn*, auxin transport is enhanced (Prayitno et al., 2006; van Noorden et al., 2006) points at a specific role for auxin transport in nodule organogenesis.

56.9 AUXIN AND RHIZOBIAL INFECTION

Over the past few years, growing evidence seems to point at a direct relation between rhizobia containing infected cells and auxin activity. Most of the research so far has focused on the involvement of auxin transport in nodule organogenesis rather than its role in infection (Mathesius, 2008; see Chapter 47). Interestingly, transcripts of *LjABCB1*, a *L. japonicus* homolog of the *Arabidopsis ABCB4* gene, were found to be nodule-specific. The promoter *GUS* fusion further showed that its expression was limited to the uninfected cells of the nodule (Takanashi et al., 2011). A chloride channel (CLC) yeast mutant (*gef1*) expressing *LjABCB1* accumulated less indole-3-acetic acid (IAA) than a control strain transformed with the empty vector control, suggesting that LjABCB1 functions as an auxin exporter. In another model symbiosis – between the actinomycete Frankia and the actinorhizal plant *Casuarina glauca*, the gene encoding the *Casuarina* homolog of the *Arabidopsis AUX1* permease *CgAux1* was found to be expressed only in infected cells of the nodule (Peret et al., 2007). Moreover, they showed that *CgAUX1* is specifically increased in infected root hairs. Using antibodies it was further shown that auxin accumulates in the infected cells of the nodule (Perrine-Walker et al., 2010). What role might auxin play in infected cells? Auxin has a well-established role in cell wall expansion/loosening and it can be expected that it may be important for the growth of the giant cells of the nodule, and possibly the redirection of root hair growth that occurs during actinorhizal infection. Although a general expression pattern of members of the *MtLAX* family was studied by *in situ* hybridization in *Medicago* (de Billy et al., 2001), it is not clear whether infection by rhizobia is similarly associated with *AUX1* expression, and studies in model legumes would provide insight into whether this feature is evolutionarily conserved.

56.10 THE ROLE OF GIBBERELLINS IN NODULATION

Gibberellins or GAs are very important plant hormones that are involved in many plant developmental processes. The GA signaling pathway (Fig. 56.2) includes perception of bioactive GA, the interaction of the GA receptor with DELLA proteins and the degradation of DELLAs leading to GA responses (Daviere and Archard, 2013). In the current model GA acts as an "inhibitor of an inhibitor," where GA derepresses DELLA proteins, which are central repressors of GA-dependent process (Harberd et al., 2009).

The function of GA in nodulation has been studied in pea (*Pisum sativum*), *L. japonicus* and *Sesbania rostrata* by using exogenous application of bioactive GA and GA biosynthesis inhibitors, examination of the expression pattern of GA-related genes, and different mutants involved in either GA biosynthesis or signaling.

GA plays a negative role during rhizobial infection and nodule formation in *L. japonicus,* which forms determinate nodules (Maekawa et al., 2009). Both the number of infection threads and the number of nodules are reduced by exogenous application of bioactive GA_3 at concentrations between 10^{-9} and 10^{-6} M. Spontaneous nodule formation in hairy root plants carrying gain-of-function of *LjCCaMK* (see Chapter 54) is also inhibited by application of GA_3. In plants overexpressing a gain-of-function allele of *LjSLY1*, which results in an increase in GA signaling, the number of nodules is reduced. It was also shown that application of GA repressed both the NF-induced and cytokinin-induced expression of *Nodulation Signaling Pathway 2* (*NSP2*) and *NIN* TFs

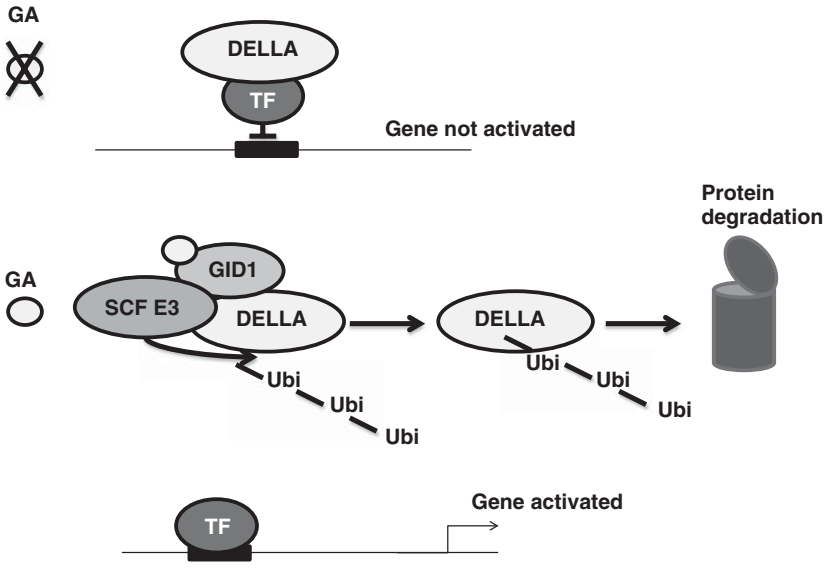

Figure 56.2 GA acts through its receptor to regulate gene expression. In the absence of GA, DELLA proteins interact with TFs (transcription factors), interfering TFs interaction with genes, and this inhibits TFs to activate genes expression. In the presence of GA, GID1 (GA receptor) binds GA, this interaction promotes GID1 interaction with DELLA proteins. The GA-GID1-DELLA complex formation stimulates substrate recognition by the SCF E3 ubiquitin ligase. This leads to DELLA proteins ubiquitination and protein degradation in the proteasome. GA-GID1-DELLA protein complex formation releases TFs which bind DNA and activate gene expression.

(Maekawa et al., 2009). It was shown that the *la cry-s* double mutant of pea exhibited a constitutive GA response due to a lack of repression by the DELLA proteins. Fewer nodules were found in the *la cry-s* mutant, suggesting that GA also inhibits nodulation in the indeterminate nodulators (Ferguson et al., 2011).

In contrast, Kawaguchi et al. found that exogenous application of GA_3 at a concentration of 10^{-7} to 10^{-4} M to *L. japonicus* could induce nodule-like structures, which arise from pericycle cell divisions (Kawaguchi et al., 1996). Similarly, exogenous application of GA_3 at concentrations between 10^{-9} and 10^{-6} to pea promotes nodule formation (Ferguson et al., 2005). Furthermore, in *L. japonicus*, enhanced GA signaling by overexpression of a gain-of-function allele of *LjSLY1* causes the formation of small nodules (Maekawa et al., 2009) and nodules from the pea GA deficient mutant *na* are aberrant and have poorly developed nodule meristems (Ferguson et al., 2011). Together these findings suggest a positive role for GA in nodulation which is consistent with its role in cell expansion, and that experiments using externally supplied GA need to be interpreted with caution. Future research directed at determining the location and timing of GA biosynthesis in nodulation, as well as additional genetic studies, are needed to better understand the role of this phytohormone.

56.11 GA IS NEEDED FOR NODULATION INVOLVING CRACK ENTRY

The *S. rostrata-Azorhizobium caulinodans* symbiosis presents an interesting case. Fernández-López et al. showed that this semi-aquatic legume can form both determinate and indeterminate nodules, depending on the conditions (Fernández-López et al., 1998). In aerated conditions *Sesbania* forms nodules by root hair curling, like most legumes. In this case indeterminate nodules are formed. In these conditions the application of GA_3 at 10^{-5} M dramatically reduced nodule number (Lievens et al., 2005), which is consistent with GA's negative role in nodulation of *L. japonicus* and pea. However, in hydroponic conditions where rhizobia invade via crack entry at lateral root bases, GA plays an opposite role. Both the NF-dependent expression pattern of a GA biosynthesis gene and pharmacological treatment with a GA biosynthesis inhibitor suggest that GA is important for infection pocket and infection thread formation; hence, the nodule primordium development during lateral root base nodulation in *S. rostrata* (Lievens et al., 2005). Interestingly, crack entry nodulation is dependent on ethylene, which in *Arabidopsis* appears to stabilize DELLA proteins and thereby antagonize GA-mediated growth (Achard et al., 2003). It seems possible then, that *Sesbania* has evolved ethylene independent GA-responses in context of waterlogged nodulation.

Such crosstalk between GA and ethylene, and possibly other plant hormones adds complexity to the investigation of GA's role during nodulation. For example, the pea *na* mutant forms fewer nodules than wild type (Ferguson et al., 2005). But later it was found that this GA deficient mutant accumulated more ethylene, which inhibits nodule formation in *na* (Ferguson et al., 2011).

What roles might GA play in nodulation? Given GA's role in cell expansion, it seems plausible that it may be involved in both infection and nodule development. During infection, root hairs expand in response to rhizobial NFs, and, in the presence of rhizobia, this leads to the development of root hair curls. The effects of GA on cell expansion in the root are dependent on auxin (Fu and Harberd, 2003), so a scenario in which these two hormones are elevated (possibly through the actions of SLs and flavonoids) and work together to facilitate root hair curling and/or infection thread growth seems plausible (Fig. 56.3).

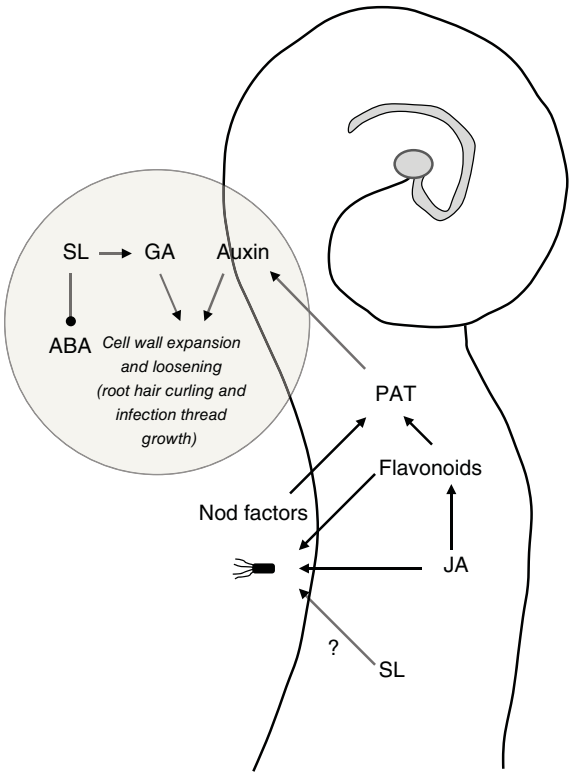

Figure 56.3 A speculative model on the role of hormones in rhizobial infection. Jasmonic acid (JA) and flavonoids induce Nod factor production in rhizobia. JA also induces the production of flavonoids. Strigolactones (SL) are also secreted into the soil, but it is unclear whether they act as a signal to rhizobia. Nod factors influence polar auxin transport (PAT) through an unknown mechanism. In seeds, SL increases gibberellic acid (GA) levels and lowers ABA levels. If GA were similarly enhanced by SL in roots during rhizobial infection, it could act together with auxin to facilitate the remodeling and growth of the root hair. In this scenario, sufficient levels of auxin would be needed within infected root hairs to promote the effects of GA.

56.12 FUTURE DIRECTIONS

The central role of hormones in rhizobial infection is unequivocal. Studies in this area will be greatly facilitated by the isolation of hormone signaling and biosynthesis mutants through forward or reverse genetics. Once the key players have been identified, it will be necessary to map out their expression during nodulation to better understand their roles and their potential interactions. Transcriptomic studies of root hairs from *S. meliloti* infected *Medicago* plants indicate that genes for auxin signaling, GA, and SL biosynthesis are enhanced, suggesting that these hormones may play key roles in the infection process. Identification of the transcriptional regulators that control these genes will help identify the intersection between the modulation of hormones and the NF signaling pathway.

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Chapter 57

Nuclear Ca^{2+} Signaling Reveals Active Bacterial-Host Communication Throughout Rhizobial Infection in Root Hairs of *Medicago truncatula*

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57.1 INTRODUCTION

The beneficial symbiotic association between nitrogen-fixing soil rhizobia and temperate legumes such as the model plant *Medicago truncatula* initiates at the root surface when the motile bacterium binds to the growing root hairs of the host. The rhizobia respond to secreted root flavonoids by synthesizing specific lipochito-oligosaccharides (LCOs) (Nod factors), which are perceived by the host and trigger a range of co-ordinated molecular and cellular responses required for both rhizobial root infection and the formation of the nodule primordium within the root cortex (for recent reviews see Oldroyd and Downie, 2008; Madsen et al., 2010; Murray, 2011; see also Chapter 51). These responses include the re-orientation of root hair tip growth leading to tip curling and the physical enclosure of the bound rhizobia between cell walls of the root hair. Interestingly, this tip-growth re-orientation can be mimicked by the localized application of Nod factors, suggesting that the curling is a direct response to Nod factor secretion at the site of rhizobial attachment (Esseling et al., 2003).

Following rhizobial enclosure within the curl, localized remodeling of the host cell wall then leads to invagination and the progressive formation of the rhizobial infection tube known as the infection thread (IT) (reviewed in Kijne, 1992; Brewin, 2004; Gage, 2004; Jones et al., 2007). The IT comprises a specialized cell wall/membrane interface which creates an apoplastic compartment into which a small number of bacteria enter from within the curled root hair. As the IT extends toward the base of the hair via a nucleus-driven tip growth mechanism the bacteria both divide and physically move in a series of successive head-to-tail files along and within the IT (Fournier et al., 2008). This complex process is discontinuous, with significant variations in IT growth rate and accompanying rhizobial colonization, and gaps are frequently present both between adjacent bacterial files and also between the growing IT tip and the leading file (Fournier et al., 2008). Once infection has been established in the root hair, a major cellular reprogramming is set in motion within the underlying cortical cells leading to the formation of the pre-infection thread (PIT) (Van Brussel et al., 1992), a polarized cytoplasmic column that traverses the cell and which serves to direct future transcellular infection. Thus, once the IT reaches the base of the root hair, apoplastic infection is continued across the underlying cortical cell, and this entire process is then repeated in

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subsequent cortical layers until membrane-bound rhizobia are delivered into the cells of the developing nodule. Finally, concomitant differentiation of the nodule tissues and of the membrane-enclosed rhizobia into bacteroids are required to generate the functional N-fixing root organ.

In addition to triggering root hair curling, Nod factors also activate the common symbiotic signaling pathway (CSSP; also knows as CSP or SYM), a key signaling pathway common to both rhizobial and arbuscular mycorrhizal symbioses (see Chapters 42, 54, 55, 110). CSSP activation leads to the transcriptional activation of symbiosis-specific genes involved in rhizobial infection/nodulation (reviewed in Kistner and Parniske, 2002; Oldroyd and Downie, 2008; Madsen et al., 2010; Murray, 2011). A unique mode of Ca^{2+} signaling lies at the heart of the CSSP, involving the initiation of sustained and regular Ca^{2+} spiking responses within both the nuclear and cytoplasmic (perinuclear) compartments (Ehrhardt et al., 1996; Shaw and Long, 2003; Oldroyd and Downie, 2006; Sieberer et al., 2009; see Chapter 54). Thus, the activation of the CSSP in root tissues can be followed *in vivo* by means of genetically engineered calcium reporters known as cameleons (Miyawaki et al., 1997), which can be expressed in specific host cell compartments (Miwa et al., 2006; Sieberer et al., 2009). The recent development of live-tissue imaging strategies for the model legume *M. truncatula* has made it possible not only to visualize the subcellular rearrangements described earlier (Fournier et al., 2008), but also to monitor cellular signaling during progressive stages of rhizobial colonization in intact roots. In this context, the use of cameleon reporters for *in vivo* calcium imaging has revealed two distinct nuclear Ca^{2+} oscillation profiles during specific stages of rhizobial infection in the root outer cortex (Sieberer et al., 2012). Low-frequency irregular Ca^{2+} spiking is initially observed in outer cortical cells preceding infection, and is associated with the characteristic cell remodeling leading to the formation of PITs. This is then followed by a rapid switch to a phase of regular high-frequency spiking concomitant with the initiation of transcellular infection of the cell. This high frequency spiking resembles that observed when exogenous Nod factors are applied to the root epidermis, suggesting that symbiotic signals generated by the rhizobia within the IT can be perceived by the host cortical cell undergoing infection (Sieberer et al., 2012). Interestingly, the high frequency spiking phase lasts for less than 1 h, and then progressively attenuates. Thus, spiking is no longer detectable during the remaining 2–3 h of transcellular infection. This very characteristic pattern of Ca^{2+} signaling is then repeated in the underlying cortical cell layers as the IT progresses toward the nodule primordium.

Having shown that cortical cell infection is accompanied by major changes in host Ca^{2+} signaling responses, we wanted to know whether similar spiking profiles could also be observed during root hair infection. This question is important because there are a number of significant differences between apoplastic infection in the epidermal and cortical cell layers. Firstly, root hair infection initiates from within the closed chamber created by tip curling around the attached rhizobia, whereas cortical infection is prepared in advance via the formation of the PIT cytoplasmic column. This means that the cell remodeling processes which precede IT initiation differ in the two tissues. Secondly, root hairs can be up to 200–500 μm in length, as compared to a typical width of only 20–50 μm for the cortical cell. And finally, as discussed earlier, root hair IT growth is a discontinuous process, alternating rapid and slow elongation rates (ranging from 1 to 12 μm/h) with occasional phases of transient growth arrest (Fournier et al., 2008). Indeed, all these characteristics have made it particularly difficult to monitor intracellular Ca^{2+} signaling throughout the various stages of root hair infection. Here we report the use of the nuclear-localized cameleon Nup-YC2.1 to follow calcium responses in root hairs during pre-infection, IT initiation, and IT elongation stages. Our results reveal similarities and differences as compared with cortical cell infection, and we discuss these in the light of the nature of the root cell undergoing rhizobial colonization.

57.2 METHODS

57.2.1 Plant Material and Rhizobial Inoculation

Agrobacterium rhizogenes-transformed composite plants of the *M. truncatula sunn-2* mutant expressing the Nup:YC2.1 cameleon (see below) were used for all the rhizobial infection studies presented in this chapter. The *sunn-2* mutant (Schnabel et al., 2005) has a supernodulation and enhanced infection phenotype, greatly facilitating the identification of root hair infection. Composite plants were grown and inoculated with *Sinorhizobium meliloti* 2011 strains constitutively expressing green fluorescent protein (GFP) in order to visualize the initiation and progression of the ITs as described in Fournier et al. (2008).

57.2.2 Cameleon Calcium Reporters and *M. truncatula* Root Transformation

To study host Ca^{2+} signaling both prior to and during rhizobial infection we made use of the 35S-driven nucleoplasmin:YC2.1 (Nup:YC2.1) cameleon that specifically targets the plant cell nucleus (Sieberer et al., 2009). Roots expressing cameleons were obtained via *A. rhizogenes*-mediated transformation (Boisson-Dernier et al., 2001) and selected according to their fluorescence levels (Sieberer et al., 2009). In addition to evaluating Ca^{2+}

responses within the nucleus, the cameleon fluorescence also facilitated the imaging of nuclear position throughout infection.

57.2.3 Microscopic Analysis of Infection Sites and Ca^{2+} Imaging in Root Cells

Infection sites were identified and imaged with a Leica TCS SP2 acousto-optical beam splitter (AOBS) confocal laser scanning microscope equipped with a long-distance 40× HCX Apo L NA 0.80 water-immersion objective. The fluorescent-labeled bacteria were imaged using fluorescence excitation at 488 nm (argon laser) and emission detection in the 500–540 nm range. Images were pseudo-colored in green using Leica Confocal Software. Fluorescence resonance energy transfer (FRET)-based ratio imaging was used for detecting the relative changes of Ca^{2+} levels corresponding to changes in YC-fluorescence of the cameleon probes (Miyawaki et al., 1997). Argon laser excitation was set to 458 nm and emissions were collected simultaneously in the 470–500 nm range (cyan fluorescent protein, CFP) and the 530–570 nm range (yellow fluorescent protein, YFP). The pinhole was set to 4 airy units (corresponding to an optical slice thickness of approximately 6 µm in order to image the entire nucleus) and images collected every 5 s. The cameleon YFP-to-CFP ratios were calculated and plotted over time using Microsoft Office Excel 2003 SP3 (Microsoft Corporation) after importing data from regions of interest drawn over cell nuclei using Leica confocal software. Relative changes in cellular Ca^{2+} levels are presented as YFP:CFP ratios (arbitrary units).

For the *Rhizobium* infection studies, data were obtained from a total of 18 experiments and the observation of 39 independent *M. truncatula* roots expressing Nup:YC2.1. Ca^{2+} spiking responses were recorded at regular intervals over 2–5 h periods during IT progression for a total of 12 independent root hairs (e.g., see Fig. 57.3). Results were obtained for an additional 17 infecting root hairs followed over shorter time periods. For the reasons discussed in the results section, recordings were difficult to obtain for Ca^{2+} signaling during the initial stages of rhizobial IT colonization (total of four root hairs: e.g., see Fig. 57.2).

57.3 RESULTS

57.3.1 *Rhizobium*-Elicited Ca^{2+} Spiking in *M. truncatula* Root Hairs Prior to Infection

In order to monitor calcium signaling responses in the host legume epidermis throughout the different phases of rhizobial infection, we exploited *M. truncatula* composite plants expressing the nuclear-localized cameleon reporter Nup:YC2.1 in *A. rhizogenes*-transformed roots, as previously described in Sieberer et al. (2009, 2012). Nuclear-associated Ca^{2+} signaling during root hair infection is difficult to monitor using conventional cytoplasmic probes, because the highly dynamic structure of the cell cytoplasm often results in low-level reporter fluorescence and limited FRET (Fournier et al., 2008; Sieberer et al., 2009). Thus, the use of the Nup:YC2.1 Ca^{2+} reporter turned out to be of crucial importance for the success of the root hair infection studies described in the following text.

M. truncatula plants were inoculated with the GFP-labeled *S. meliloti* strain 2011 and the initial pre-infection stages of the interaction were monitored using light microscopy. *Rhizobium*-elicited root hair curling was observed in growing hairs from 12 h post-inoculation onwards, and Figure 57.1a illustrates three consecutive stages of tip curling in an individual root hair. Observations of numerous such curlings suggest that a single bacterium attached close to the hair tip is sufficient to activate tip growth re-orientation toward the site of attachment, and that the entire curling around the attached *Rhizobium* is completed within approximately 4–5 h (results not shown).

It has already been shown that Ca^{2+} spiking in legume root hairs is triggered within minutes following rhizobial inoculation and is dependent on Nod factor synthesis (Wais et al., 2002; Harris et al., 2003). Our live-tissue confocal FRET imaging experiments performed with cameleon calcium reporters have revealed that the capacity of growing root hairs to respond to rhizobial signals is maintained for many hours post-inoculation. Furthermore, the results presented in Figure 57.1a show that regular high frequency spiking is still present in root hairs throughout tip curling and we presume that this very characteristic response is also the result of the perception of rhizobial-secreted Nod factors.

Although root hair curling is a relatively rapid process resulting in the physical enclosure of the rhizobia within a space surrounded by host cell walls (referred to hereafter as the "infection chamber"), continuous live-cell imaging has shown that there is always a lengthy delay (12–20 h) before the initiation of IT growth from within the curl. Using a variety of cellular markers, we now have strong evidence that major host cell wall remodeling takes place within the infection chamber during this period (Fournier et al., 2015). Strikingly, our *in vivo* imaging experiments have revealed that Ca^{2+} oscillations can no longer be detected in the majority of fully curled root hairs, and in the remaining curled hairs spiking is both infrequent and irregular (Fig. 57.1b,c). In contrast, non-deformed growing root hairs present in the vicinity of the curled hairs continue to show typical Nod factor-elicited spiking. This suggests that once the closed infection chamber has been formed, the root hair temporarily loses its capacity

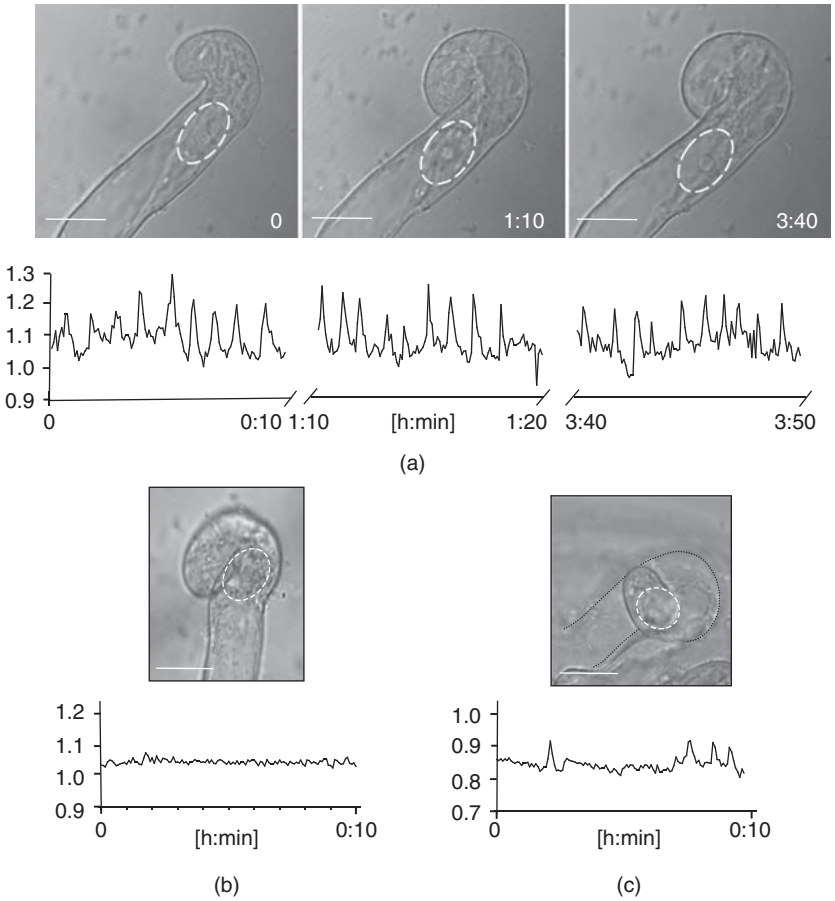

Figure 57.1 Ca^{2+} spiking in *M. truncatula* root hairs during different stages of rhizobial-elicited hair curling. (a) Three consecutive stages of root hair curling over a 4 h period are illustrated, and underneath each image the corresponding high frequency Ca^{2+} spiking profiles observed within the hair nucleus (dashed outlines). (b,c) Nuclear Ca^{2+} spiking is severely attenuated in root hairs once the 360° curl is completed and the rhizobia fully enclosed in a chamber surrounded by host cell walls. The fact that the nuclei are positioned in very close proximity to the infection chamber shows that the loss of spiking is not due to the migration of the nucleus away from the entrapped rhizobia. Scale bars = 10 μm.

to respond to rhizobial Nod factor LCO signals and to activate the CSSP.

57.3.2 Infection Thread Initiation in Root Hairs Correlates with Reactivation of Ca^{2+} Spiking

Throughout root hair curling and the formation of the infection chamber, rhizobial multiplication within the enclosed curl leads to the formation of a microcolony generally comprising less than 20 bacteria. The initiation of the IT corresponds to the localized invagination of the host membrane that surrounds the infection chamber toward the base of the root hair, following a path which is defined by the broad cytoplasmic column linking the chamber to the migrating cell nucleus (Fournier et al., 2008). The bright-field image in Figure 57.2a illustrates a root hair just prior to IT initiation, and shows the relative positioning of the cell nucleus and cytoplasmic bridge to the infection chamber within the curl. The corresponding fluorescence confocal image (Fig. 57.2b) reveals both the GFP-labeled bacterial microcolony present within the chamber and the intense fluorescence in the nucleus corresponding to the Nup:YC2.1 cameleon.

Unfortunately, it is extremely difficult to anticipate the precise moment of IT initiation within the curled root hair for two reasons. Firstly, as stated earlier, there is a lengthy and variable period of time associated with the cell wall remodeling, which takes place within the infection chamber. Secondly, a majority of curled *M. truncatula* root hairs do not progress beyond this stage and never initiate ITs. The reason for this is unclear, but has also been reported for other legume plants (e.g., Callaham and Torrey, 1981 in the case of clover root hair infection). As a result, we have only been able to observe a limited number of IT initiation events, of which a typical example is shown in Figure 57.2c. A comparison with Figure 57.2b shows that rhizobia have entered the newly formed IT during the 3 h period between the two images. When the Ca^{2+} status in the root hair nucleus was evaluated using the fluorescent cameleon reporter, well-defined spiking was present both prior to and immediately following rhizobial entry into the newly formed IT (Fig. 57.2). These findings were confirmed for at least three other root hairs, and lead to the proposition that Ca^{2+} spiking is reactivated in the root hair nucleus at a stage which accompanies IT initiation.

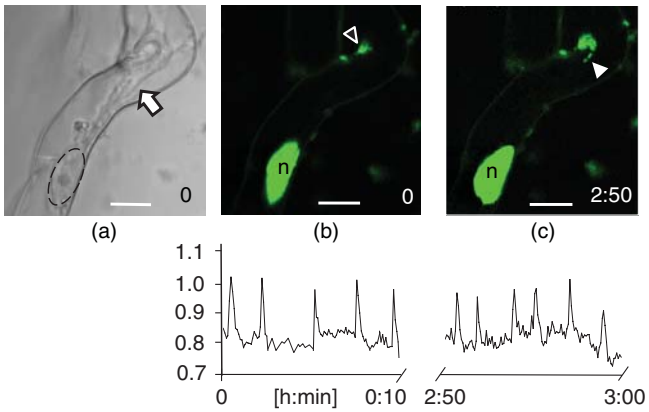

Figure 57.2 Reactivation of Ca^{2+} spiking during infection thread initiation. (a,b) Bright field (a) and corresponding fluorescence (b) images of a root hair during IT initiation at a stage preceding rhizobial entry. The position of the nucleus is outlined in (a) and indicated by "n" in (b), the arrow in (a) indicates the position of the broad cytoplasmic bridge linking the nucleus to the infection chamber and the dark arrowhead in (b) shows the position of the bacterial microcolony. The Ca^{2+} spiking profile shown underneath image (b), derived from FRET-based confocal imaging of the nuclear-localized fluorescent cameleon Nup:YC2.1, shows that spiking has been reactivated at this pre-infection stage. (c) Sustained spiking is still present in the nucleus (n) when the first fluorescent rhizobia can be seen entering the IT compartment (white arrowhead) approximately 3 h later. Scale bars = 10 µm.

57.3.3 Ca^{2+} Signaling is Associated with Active Phases of IT Growth during Root Hair Infection

As outlined in the introduction, a similar *in vivo* imaging approach previously revealed that the initiation of IT growth in the underlying root cortical cell tissues is concomitant with a rapid switch to high frequency nuclear Ca^{2+} spiking in the target cell (Sieberer et al., 2012). However, this spiking response is only of limited duration (<1 h), and is absent during the remaining period of IT growth across the cortical cell. For this reason, it was important to determine to what extent Ca^{2+} spiking is maintained throughout the lengthy period of infection in the root hair, especially because IT growth within this specialized epidermal cell is a highly irregular process, alternating between periods of rapid and slow tip growth and occasional temporary arrest (Fournier et al., 2008).

The four consecutive images presented in Figure 57.3 cover a total period of 3 h and perfectly illustrate the variable nature of IT growth within an individual root hair. During the 40 min period between the first two images (Fig. 57.3a,b), the rhizobial file has progressed at an average rate of 12.6 µm/h. This corresponds to the maximal rate of IT progression observed in *M. truncatula* root hairs (Fournier et al., 2008). Nuclear Ca^{2+} spiking responses can be clearly observed both at the beginning and the end of this phase of

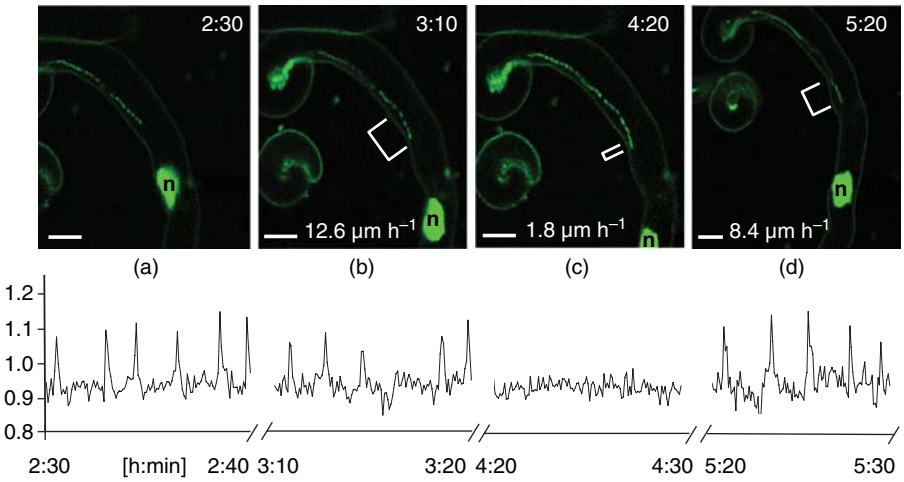

Figure 57.3 Ca^{2+} spiking in infected root hairs correlates with phases of pronounced IT growth. (a–d) The four confocal images covering a total period of 3 h illustrate typical discontinuous IT growth within a root hair which was monitored regularly over a 5 h 30 min period. The fluorescent-labeled nuclei are marked by "n" and the individual fluorescent rhizobia can be identified within the advancing thread. The distance moved by the leading bacteria within the IT between successive images is indicated by the bracket and the corresponding average speed of rhizobial colonization over these periods (40, 70, and 60 min respectively) is indicated at the bottom of images (b–d). The 10 min-duration Ca^{2+} spiking profiles which correspond to each time-point are shown underneath each image and reveal that spiking is present during this entire period of IT growth with the exception of the 4 h 20 min time-point (c). It is striking that this absence of spiking correlates with the slowest phase of bacterial progression (7 and 5× slower than in b and d respectively), most likely corresponding to a slow phase of IT tip growth. Scale bars = 10 µm.

rapid bacterial colonization. However, during the following hour (Fig. 57.3b,c), file progression has slowed to an average of only 1.8 μm/h, and it is striking that there is a concomitant loss of nuclear spiking at the end of this period. During the final hour of imaging, the bacterial colonization rate re-accelerates (Fig. 57.3c,d) to reach an average speed of 8.4 μm/h and this increased growth rate is accompanied by a reactivation of Ca^{2+} spiking within the root hair nucleus. Time-course observations performed on at least 10 other *M. truncatula* root hair infections (see Section 57.2) have revealed a similar positive correlation between high IT growth rates and active nuclear Ca^{2+} signaling. Finally, it should be underlined that spiking was never observed in an infected root hair in which the IT had permanently stopped growth. In conclusion, these findings clearly demonstrate that, in contrast to cortical cell infection, the Ca^{2+} spiking response in root hairs is not shut down shortly after IT initiation and the likely significance for this difference will be discussed below.

57.4 DISCUSSION

57.4.1 Studying Calcium Signaling in Root Hairs Throughout Apoplastic Rhizobial Infection

For the majority of temperate legumes, including the two model species *M. truncatula* and *Lotus japonicus*, initial *Rhizobium* root entry occurs via a unique cellular process involving the creation of a novel apoplastic compartment within tip-growing root hair cells. In order to obtain information about the dynamics of microbe-host signaling throughout root hair infection we have used confocal microscopy to monitor Ca^{2+} signaling in *M. truncatula* root hairs during the various stages which both precede and accompany IT growth. The nuclear-expressed cameleon Nup:YC2.1 has proved to be an ideal calcium reporter for these studies, greatly facilitating time-lapse observations during the highly irregular phases of IT growth along the root hair.

Following rhizobial inoculation, at least two morphologically distinct pre-infection phases can be distinguished in *M. truncatula* roots. Firstly, root hair tip curling around attached rhizobia is observed approximately 12–24 h post-inoculation. The 360° tip-curling which generates the so-called shepherd's crook is relatively rapid and usually completed within 4–6 h (Fig. 57.1). However, IT initiation from within the closed curl was only observed in our experiments following an additional 12–20 h. As commented earlier, we now have strong evidence that major host cell wall remodeling is taking place around the enclosed rhizobia during this second pre-infection phase (Fournier et al., 2015). This phenomenon is not limited to *M. truncatula*, since Callaham and Torrey (1981) previously reported, using light microscopic observation, that ITs are only initiated in clover root hairs 12–16 h after the completion of root hair curling. Together, these findings underline the fact that IT initiation is not simply the result of a switch in polarity from the outward tip-growing root hair to the inward tip-growing IT, as has frequently been suggested (e.g., Jones et al., 2007).

The results presented in Figure 57.1a show clearly that high frequency nuclear Ca^{2+} spiking is present in *M. truncatula* root hairs undergoing tip-curling. This spiking profile is very similar to that observed in root hair nuclei in response to purified Nod factors (Sieberer et al., 2009), suggesting that these cells are responding to the rhizobia-secreted LCO signals. It was previously shown that Nod factor-like Ca^{2+} spiking in *M. truncatula* root hairs could be elicited within minutes following *S. meliloti* inoculation (Wais et al., 2002), and indeed our time-lapse imaging has shown that this highly characteristic spiking is maintained in actively tip-growing root hairs for many hours following inoculation, including in those adjacent to fully curled hairs. It is therefore unclear whether the spiking response present in tip curling hairs is being elicited by LCOs generated locally by the attached rhizobia or by LCOs that have accumulated in the medium surrounding the root following inoculation. Whatever the source of rhizobial Nod factors, we can conclude that root hairs undergoing 360° curling are fully responsive to these symbiotic signals.

In contrast, root hairs that have completed curling to form what we have termed the "infection chamber" appear to have a modified capacity to respond to the rhizobial elicitors, as nuclear spiking is either totally absent or severely attenuated in these hairs (Fig. 57.1b,c). The limited spiking in these cells is unlikely to be due to a lack of exogenous Nod factors as adjacent tip-growing root hairs show normal Ca^{2+} spiking responses. We therefore favor the hypothesis that this transient loss of LCO responsiveness is a consequence of the cell wall/membrane remodeling that accompanies this particular pre-infection phase.

57.4.2 Nuclear Ca^{2+} Spiking is Reactivated and Maintained Throughout IT Growth Phases within the Root Hair

For the reasons described earlier it turned out to be particularly difficult to capture the earliest stages of IT formation in the *Medicago* root hair. Nevertheless, our data-set (e.g., Fig. 57.2) indicates that sustained Ca^{2+} spiking has been reactivated prior to and during the entry of rhizobia into the newly created apoplastic compartment. This is consistent with the results obtained previously for outer cortical cell infection, where regular high frequency spiking is rapidly switched on during the earliest infection stages

(Sieberer et al., 2012). Of course, additional experiments using cellular markers that specifically label the IT cell wall/membrane interface will now be required to determine precisely when Ca^{2+} spiking is activated with respect to IT initiation in the root hair.

As shown previously (Fournier et al., 2008), subsequent IT extension within the root hair shaft is a non-regular process involving a complex interplay between nuclear migration, the dynamics of the cytoplasmic column connecting the nucleus to the growing IT tip, and the progressive bacterial colonization of the newly-formed thread. By monitoring IT growth in a number of root hairs over lengthy 2–3 h periods and in different parts of the root hair, we have been able to draw a number of preliminary conclusions. Firstly, nuclear Ca^{2+} spiking can be observed throughout different stages of IT growth within the root hair, a process which can last for up to 20 h. Thus, in contrast to the situation for outer cortical cell infection (Sieberer et al., 2012), spiking in root hairs is neither attenuated nor switched off within the 1–2 h period that follows IT initiation. Secondly, the spiking response appears to vary as a function of the IT growth rate, with periods of active spiking alternating with a partial or even complete loss of nuclear Ca^{2+} oscillations. This is well illustrated in Figure 57.3, where a transient loss of spiking in the root hair nucleus is concomitant with a major reduction in the rate of rhizobial progression within the thread. Similar transient spiking arrests were observed in other root hair infections monitored over several hour periods, and in each case this coincided with a phase of reduced bacterial progression. Although we assume that these periods of slow rhizobial progression correlate with phases of reduced IT tip-growth, additional studies using IT-specific markers will be required to determine whether the absence of nuclear spiking is directly linked to reduced rates of IT extension and/or other parameters such as the IT tip-to-nuclear distance (Fournier et al., 2008). This information is important if we wish to understand the precise relationship between the activation of the Ca^{2+}-mediated host signal transduction pathway and sustained IT tip growth in the root hair.

In conclusion, these findings underline the requirement for active rhizobial-host signaling throughout root hair infection. As for cortical cell infection (Sieberer et al., 2012), the regular sustained Ca^{2+} spiking observed within the root hair nucleus (Figs. 57.2 and 57.3) resembles Nod factor-induced spiking profiles and this is therefore totally consistent with the perception of rhizobial signals by plant receptors located in the IT membrane. Indeed, the well-characterized infection-related MtLYK3 "entry" receptor (Limpens et al., 2003; Smit et al., 2007) recently localized to the IT periphery (Haney et al., 2011) would be an excellent candidate for perceiving these symbiotic LCOs.

57.4.3 Different Modes of Ca^{2+} Signaling during Root Hair Versus Cortical Cell Infection

The findings reported here have revealed a striking difference between root hair and cortical cell infection in relation to the maintenance of Ca^{2+} spiking throughout transcellular IT growth. Could this reflect fundamental differences in the mode of IT progression between the two cell types? In the case of variable-length root hairs, the infection compartment construction depends both on the relative positioning of the migrating nucleus and the organization of the interconnecting cytoplasmic bridge. The highly variable nature of these two parameters no doubt contributes to the characteristically irregular IT growth dynamics observed in root hairs. In contrast, infection of the underlying cortical cells is programmed in advance via the formation of the transcellular PIT, which guides subsequent IT growth. We can thus speculate that the continued perception/transduction of symbiotic signals generated by the colonizing rhizobia within the growing IT is required for successful root hair infection, whereas the activation of this same signaling pathway leading to Ca^{2+} spiking is only necessary for the initial stages of cortical infection.

In a recent article, Liao et al. (2012) have described a novel and particularly interesting mutation in the *L. japonicus CCaMK* gene (*ccamk-14*), which results in the premature abortion of rhizobial infection in the host outer root cortex. CCaMK is the nuclear-localized calcium and calmodulin-dependent kinase which plays a central role in the CSSP pathway decoding intracellular oscillatory Ca^{2+} signaling elicited by rhizobial Nod factors (Lévy et al., 2004; Gleason et al., 2006; see Chapter 54). *In vitro* studies indicated that the *ccamk-14* mutation (S337N) occurs at a putative auto-phosphorylation site within the CaM-binding domain of CCaMK (Liao et al., 2012). Furthermore, the authors have provided good evidence that the auto-phosphorylation of S337 interferes with CaM binding and that this is involved in the negative regulation of CCaMK activity. Finally, an intriguing feature of the *ccamk-14* mutation is that while rhizobial infection aborts in the outer cortex, infection appears to proceed normally in the overlying root hairs. This suggests that the negative regulation of CCaMK and the consequent inactivation of the CSSP pathway is essential for the successful completion of cortical infection but not for epidermal root hair infection. By analogy, the differences that we have observed in relation to Ca^{2+} spiking between the two outer root tissues in *Medicago* could indeed be the result of different requirements for an active CSSP once infection has been initiated. Future research now needs to be focused on the possible interplay between the negative regulation of Ca^{2+} signaling and the negative autoregulation of CCaMK during cortical cell infection in both *Medicago* and *Lotus,* with the goal of

understanding why the inactivation of the CSSP is crucial for successful cortical infection.

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Chapter 58

A Pectate Lyase Required for Plant Cell-Wall Remodeling During Infection of Legumes by Rhizobia

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58.1 INTRODUCTION

In order to infect legume roots and nodules, rhizobia induce the plants to form infection threads (ITs), which are established by the initiation of an inward growth of the plant cell wall and membrane. The resulting IT structure resembles an intracellular tube that is lined with newly synthesised plant cell wall and membrane (see Chapter 57). Within the space lined by the plant cell wall there is an apoplastic-like compartment that contains glycoproteins, carbohydrates, and extensins within the matrix (Brewin, 2004).

Rhizobia grow and have some sliding movement near the growing tip of ITs (Fournier et al., 2008) and the elongation of the ITs is usually preceded by nuclear movements that direct their growth (Fahraeus, 1957; Nutman, 1959; Fournier et al., 2008; see Chapter 57). In most temperate legumes, initiation of IT growth occurs from infection foci, formed after the root hairs grow back on themselves entrapping rhizobia that divide in the closed pocket that is formed. However, in some legumes infection does not occur via root hairs, but can be initiated from pockets of bacterial growth formed after rhizobia colonize intercellularly (Boogerd and van Rossum, 1997; Capoen et al., 2010). Indeed it has recently become apparent that, even in legumes in which root-hair infection is the norm, nodule infections can be initiated via a pathway that does not involve epidermal ITs (Karas et al., 2005; Madsen et al., 2010)

Irrespective of whether ITs are initiated in root hairs or in deeper layers of the root, the initiation of IT growth requires remodeling of the plant cell wall. Even before rhizobia had been identified as the infecting micro-organisms in legume nodules, growth of ITs had been observed and it had been deduced that there must be some degradation of plant-cell walls to allow the infection to occur (Ward, 1887).

As the IT initiates and grows down through the root hair, new cell-wall material must be synthesised and this will require delivery of cell-wall synthesis enzymes, structural proteins and cell wall precursors to the growing IT tip. As the growing IT approaches the base of the root-hair cell, the newly synthesized IT cell wall must become cross-linked with the existing root-hair cell wall. In parallel, for the IT to cross between the root-hair cell and the next sub-epidermal cell, there must be a localized degradation of the cell wall at the base of the root hair and opposing sub-epidermal cell wall. Prior to this cell-wall loosening and the arrival of the growing IT tip, the cytoskeleton of the sub-epidermal cell is rearranged and establishes the direction of growth of the IT (van Brussel et al., 1992).

In this short review we will consider the events that will be required to occur at the cell wall to initiate the growth

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of ITs and their continued growth through adjacent cells to allow nodule infection to occur. This requires localized cell wall loosening/degradation that is coordinated with new cell-wall synthesis.

58.2 CELL WALL COMPONENTS

To understand what may be required to remodel the primary cell walls during initiation of infection, it is important to understand the principle components. Cellulose microfibrils form a structural scaffold that is tied together and given rigidity by crosslinks to xyloglucans and pectins. Xyloglucans can bind to and be entrapped within the cellulose microfibrils, acting as crosslinking tethers that stabilize the cellulose scaffold (Cosgrove, 2005). Pectins come in different major forms all based on α1–4 linked galacturonic acid. These forms include homogalacturonan (which can constitute up to 60% of the pectin in primary cell walls and contains some residues that are methyl esterified), and more complex substituted forms of homogalacturonan including the rhamnogalacturonans RG-I and RG-II, xylogalacturonan and apiogalacturonan (Mohnen, 2008). The different pectins confer physical strength to the cell wall and contribute to the formation of a barrier to the external environment, especially with regard to strengthening the plant cell wall (Caffall and Mohnen, 2009).

When considering infection by rhizobia, it may useful to consider which cell-wall components are targeted by cell-wall-degrading enzymes secreted by plant pathogens. Based on the diversity and range of virulence-associated enzymes that are involved with pectin degradation (pectate lyases, polygalacturonases, and pectin methyl esterases), it is clear that this polymer is the main target for degradation by pathogens (Cantu et al., 2008). Although both pectate lyases and polygalacturonases can degrade the same substrate, they are very different enzymes; polygalacturonases hydrolyse the glycosidic bond, whereas pectate lyases catalyse a β-elimination reaction that cleaves the glycosidic bond. In addition to these pectin-degrading enzymes, xylanases are important for plant cell-wall degradation by some pathogens (Rajeshwari et al., 2005; Brito et al., 2006). Necrotrophic pathogens tend to macerate the plant cell wall and usually have higher numbers of genes encoding cell-wall degrading enzymes than biotrophic fungi. For example, the genomes of the necrotrophic pathogens *Botryris cinerea* and *Sclerotia sclerotiorum* genomes appear to be specially adapted for pectin decomposition (Amselem et al., 2011).

Biotrophic fungi such as *Puccinia graminis* gain access to plant cellular nutrients by locally degrading the plant cell wall adjacent to the appresorium; the associated fungal penetration leads to the production of the haustorium complex which is a fungal intracellular feeding structure (Ehrlich and Ehrlich, 1962). The biotrophic ergot fungus *Claviceps purpurea* has two polygalacturonases that are needed for virulence and growth between cells (Oeser et al., 2002). Such localized cell-wall degradation by biotrophic pathogens points to a specific role for pectin degradation in locally penetrating the plant cell wall; possibly this can be seen as being analogous to the cell-wall remodeling that occurs during rhizobial infection. However, the products of pectin degradation by plant pathogens activate a plant defence cascade (Kohorn et al., 2009) and such a defence response could be a significant problem during rhizobial infection. During cell division, plant cells must locally degrade some pectins and probably other cell-wall components and this seems usually to occur without the strong induction of plant defences (otherwise plant defences would constantly be activated during cell division). So it seems likely that plants have evolved ways of inducing some cell-wall degrading enzymes to allow remodeling of their cell walls without inducing defence responses. Harnessing such plant enzymes during the initiation of infection by rhizobia would be an appropriate way of promoting cell-wall remodeling to allow IT growth initiation without inducing plant defences from products of limited cell-wall degradation.

58.3 INFECTION THREAD INITIATION

Initiation of ITs requires rhizobially produced Nod factors and the consequent activation of the nodulation signaling pathway in legumes (Murray, 2011). Mutations in Nod-factor-receptor genes block Nod-factor-induced responses such as root-hair deformation and perinuclear calcium spiking. However, mutations that block the calcium spiking signaling pathway do not block all root hair responses: for example, mutants blocked in the Nod-factor-induced calcium-spiking pathway retain some root-hair deformation, although in those mutants, the characteristic full root-hair curling that causes rhizobial entrapment did not occur (Miwa et al., 2006). It is the bacterial micro-colonies that develop after the rhizobia are entrapped by the curled root hairs that form infection foci; these foci of entrapped rhizobia define where the plants will initiate the growth of ITs.

The sites of initiation of IT growth in root hairs and the locations where ITs will extend into cortical cells are marked by nuclear relocalisation to the vicinity of new sites of cellular penetration (see Chapter 57). The cytoskeletal rearrangements set up by the nucleus (Timmers, 2008) result in structures called pre infection threads (PITs) and these can be induced by rhizobiallymade Nod factors (van Brussel et al., 1992). Different genes contributing to changes in the assembly of the cytoskeleton have been identified as being required for IT growth, because mutations in these genes block early infection events. Thus, mutations in *NAP1* and *PIR1* block infection; they encode components of the major class 1 actin

nucleation promoting complex (Yokota et al., 2009; Miyahara et al., 2010). Similarly, mutation of the *ARPC1* gene blocks infection; *ARPC1* encodes actin- related protein component 1, which is a component of the major nucleator of Y-branched actin filaments in plants (Hossain et al., 2012).

The rearranged positioning of the actin cytoskeleton at likely infection foci provides a framework, which could be used to deliver plant-cell-wall degradation and cell-wall synthesis enzymes to localized sites. Vesicle trafficking can be directed along the actin cytoskeleton (Samaj et al., 2006) and so there is the potential to deliver glycanases, cellulases, pectinases, polygalacturonases and pectin esterases to the localized region of the cell wall adjacent to the cytoskeletal components that contribute to the PIT. Specific exocytotic-vesicle-associated membrane proteins (VAMPs) have recently been identified as being important for the delivery of proteins to developing symbiosomes (Ivanov et al., 2012). Although no such symbiosis-specific pathway has yet been identified for targeting of plant cell-wall degrading enzymes to the sites of IT initiation, it seems likely that some such targeting must occur.

What is the evidence for a requirement for plant-made cell-wall degradation localized at the sites of initiation of infections by rhizobia? Electron microscopy of such sites revealed localized degradation of the plant cell wall close to rhizobial infection sites. These sites of infection initiation and cell-wall softening were associated with large numbers of plant-made vesicular bodies (Ridge and Rolfe, 1985). At these sites of infection, the rhizobia appeared to be appressed against the cell wall (Ridge and Rolfe, 1985), suggesting that the bacteria may exert a pressure that distorts the cell wall. However, once the IT starts to grow, it is evident that there is frequently a bacteria-free zone near the IT tip, demonstrating that the bacteria need not be adjacent to the growing tip during its progression (Fournier et al., 2008; see Chapter 57). This is consistent with a model in which IT growth and extension is controlled and maintained by the plant. However, after PIT formation, fullyformed ITs are not formed unless bacteria are growing within them implying that IT growth and elongation must be very closely coordinated with rhizobial growth.

It is now evident that one plant enzyme required for infection is a pectate lyase that is specifically induced in legumes in response to the signaling cascade induced by the rhizobially made Nod factors (Xie et al., 2012). Mutation of this Nod-factor induced pectate lyase gene (*NPL*) in *Lotus japonicus* resulted in the blocking of rhizobial infection at the sites of infection foci in root hairs as illustrated by the blocked infection in Figure 58.1b compared with the normal infection shown in Figure 58.1a. The *npl* mutant produced

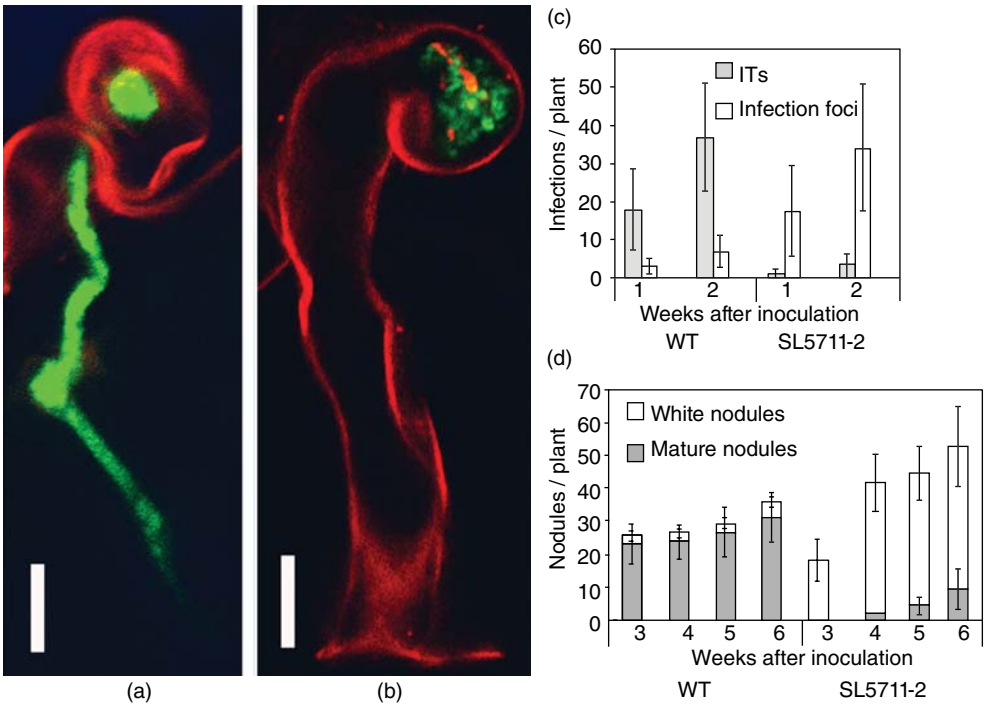

Figure 58.1 Nodulation and infection defects of a *Lotus japonicus* nodulation pectate lyase (*npl-1*) mutant. Panels (a) and (b) show confocal laser scanning fluorescence microscope images of root hairs of WT (a) and the npl-1 mutant SL5711-2 (b) seedlings inoculated with *M. loti* carrying GFP. An infection focus without an associated IT is shown in a root hair of the *npl-1* mutant. Panel (c) shows the average numbers (±SE) of ITs and infection foci in WT and the *npl-1* mutant SL5711-2, 1 and 2 weeks after inoculation with *lacZ*-marked *M. loti*. Panel (d) shows the average numbers (±SE) of mature nodules and white nodules on WT and *npl1-1* mutant scored 3–6 weeks after inoculation (±SE N > 20). Scale bars in (a) and (b) are 12 µm. (*Source*: Adapted from (Xie et al., 2012).)

many infection foci, most of which did not progress to form ITs (Fig. 58.1c). In a few cases infections did occur in the mutant but these appeared to be arrested when the IT reached the next cell wall. The net result was that the mutants produced white nodules, most of which were uninfected (Fig. 58.1d).

The mutated gene was identified by positional cloning and DNA sequencing revealed that one *L. japonicus npl* mutant had a predicted premature stop codon and the other had a missesnse mutation in a region conserved in other pectate lyases. The wildtype and missense mutant form of the pectate lyase were purified, whereas the WT NPL was active on both polygalacturonic acid and its methyl esterified form (pectin), the missense mutation abolished most or all of this activity (Xie et al., 2012).

The *NPL* gene was strongly induced in roots by Nod factors produced by *Mesorhizobium loti* and this induction was blocked by mutations in genes in the Nod-factor signaling cascade (Fig. 58.2a). Normal induction of *NPL* also requires the NIN transcription factor which bound to the *NPL* promoter (Xie et al., 2012). To assay the pattern of *NPL* expression in roots, the promoter of *NPL* was fused to the GUS (β-glucuronidase)-encoding reporter gene (Xie et al., 2012); this gene fusion was strongly induced in curled root hairs inoculated with *M. loti* (Fig. 58.2b, c) and this pattern is similar to the pattern of *NIN* induction (Kosuta et al., 2008).

The *Medicago truncatula* orthologue of *L. japonicus NPL* was identified and it was one of two very similar pectate lyase genes that are closely linked on *M. truncatula* linkage group 3 (Xie et al., 2012). As shown from the phylogeny in Figure 58.3, these two predicted pectate lyases cluster together with *L. japonicus* NPL and two other predicted pectate lyases from *Glycine max*. This group forms a phylogenetically distinct subgroup that does not contain a representative pectate lyase from *Arabidopsis thaliana*.

It has long been recognized that the initiation of legume infection was associated with increased production of clover root-hair polygalacturonase (Ljunggren and Fahraeus, 1959, 1961). Previously a rhizobially-induced polygalacturonase gene (*MsPG3*) was identified in *Medicago sativa*; this gene was strongly induced in nodule primordia and in the infection zone of nodules (Munoz et al., 1998). It was not reported if this gene was expressed in root hairs, but its pattern of expression seems to be somewhat different from that seen with the *L. japonicus NPL* gene, indicating that there may be different pectin/polygalacturonic acid degrading enzymes induced during nodulation. It was reported (Xie et al., 2012) that at least two polygalacturonases are also induced during nodulation, based on an analysis of gene expression in the *M. truncatula* Gene Atlas Expression (MtGEA) database (Benedito et al., 2008).

Taking into account the structure of the plant cell wall, it seems likely that other polysaccharide degradation enzymes will be induced by plants. Pectin methylesterase genes

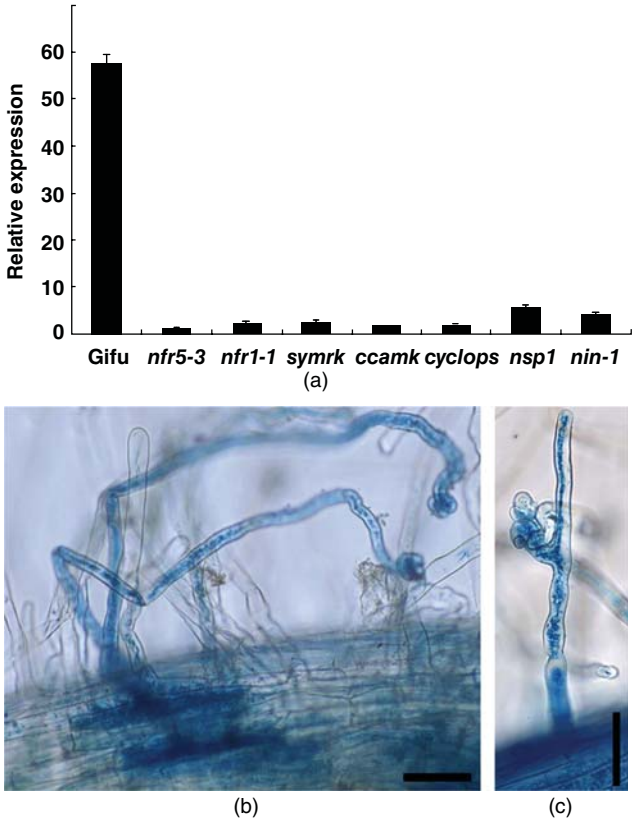

Figure 58.2 *LjNPL* induction requires Nod-factor signaling and *NIN*. Panel (a) shows induction of *LjNPL* by M. loti Nod factor (10 nM) relative to untreated controls, measured by quantitative RT-PCR using RNA isolated from WT and the nodulation mutants indicated. Panels (b) and (c) show *Agrobacterium rhizogenes*-induced hairy roots on chimaeric seedlings of *L. japonicus* WT transformed with the promoter region of *LjNP* upstream of the β-glucuronidase gene. The roots were inoculated with *M. loti* and 8 days after inoculation the roots were stained for 3 h using X-gluc as described (Xie et al., 2012). The curled root hairs stained strongly. The scale bars are 50 μm.

have been identified as being specifically induced during nodulation (Lievens et al., 2002); based on the analysis of the *M. truncatula* gene atlas database a pectin methylesterase was also found to be induced during nodulation (Xie et al., 2012).

Our analysis of the *M. truncatula* GeneAtlas database (http://mtgea.noble.org/v3/) identified several predicted glycanases that are expressed during nodulation as are some predicted cellulases. However, none of these genes has been identified as having the highly specific pattern of expression of the *M. truncatula NPL* gene. This of course does not necessarily mean that such enzymes do not function in legume infection. The regulation of other cell-wall degrading enzymes could for example be less specific than that of NPL, or their activity could depend more on the regulation of their delivery, for example, via targeting to a vesicle-dependent exocytotic pathway.

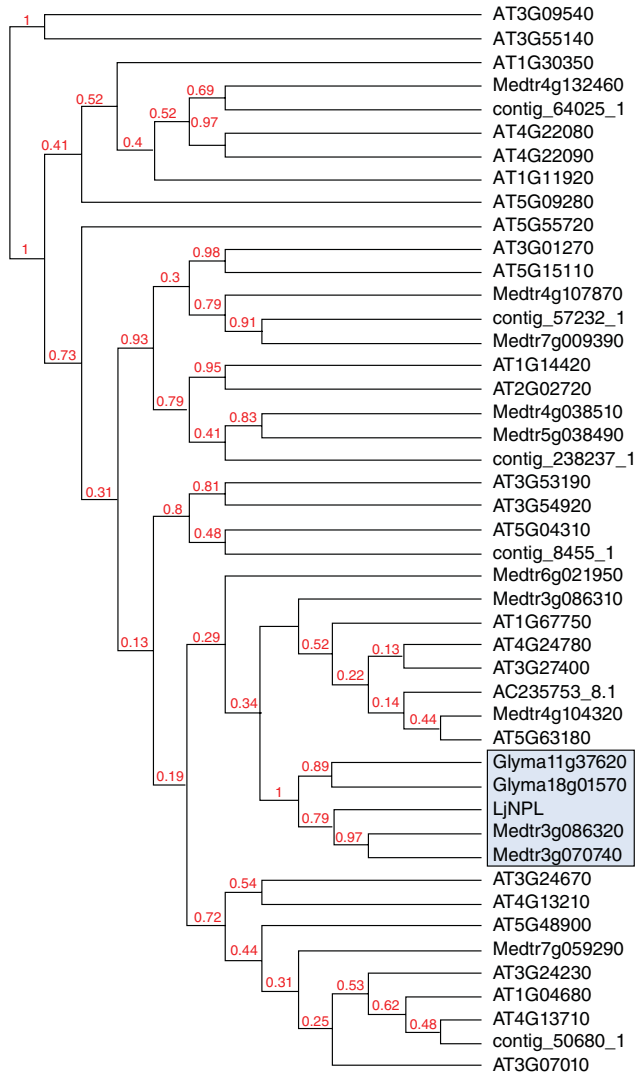

Figure 58.3 Identification of a legume-specific clade of pectate lyases. The phylogeny shows LjNPL and pectate lyase sequences from *Arabidopsis thaliana* and *Medicago truncatula*. *A. thaliana* protein sequences (AT) are from TAIR (http://www.arabidopsis.org/); *M. truncatula* proteins (prefixed 'Medtr' 'AC' or contig) are predicted from IMGAG version 3.5 (http://www.jcvi.org/cgi-bin/medicago/annotation.cgi). The pectate lyases from soybean (SoyBase http://soybase.org/; Glyma11g37620 and Glyma18g01570) are included because they are symbiotically induced; they cluster with LjNPL and with *M. truncatula* Medtr3g086320 (nodulespecific isoform), forming a distinct clade with strong support (blue box). The phylogenetic tree is as presented previously (Xie et al., 2012) and was constructed using a maximum likelihood method, assessing the reliability of branches using the bootstrapping method (100 bootstrap replicates).

It is possible that targeted degradation caused by pectate lyases and polygalacturonases, along with changes in the methylation status of pectin induced by pectin methylesterase, could allow sufficient cell-wall softening that could permit initiation of infection. One way in which this could occur is by the growing colonies of rhizobia in infection foci pushing against the weakened cell wall, followed by cell wall and membrane synthesis coordinated via the rearranged cytoskeleton. The observation that with the *L. japonicus npl* mutant some infections appear to initiate after unusually large numbers of bacteria accumulate in infection foci (Xie et al., 2012) gives some support to this hypothesis if one takes the view that larger accumulations of rhizobia can exert greater pressure. Possibly, such stretching of the cell wall could activate localized cell wall loosening that is associated with the growth of new cell walls. Cell walls expand their cell walls by a carefully controlled process of controlled 'polymer creep' in which the actions of expansins and glucosylases allow movements of polymers that can allow the cellulose microfibrils to move apart (Cosgrove, 2005). Induction of an expansin gene has been observed to be induced in *Melilotus alba* within hours of rhizobial inoculation (Giordano and Hirsch, 2004).

The pectin is clearly one of the components of the wall matrix that must be cleaved, but it is not yet clear what other degradative enzymes will be required. It is evident that the normal process of cell wall synthesis (Cosgrove, 2005) must be activated in order to extend the IT. In some regards, making the connection between the existing plant cell wall and the initiating IT cell wall may be analogous to the process by which a newly synthesised cell wall in a dividing plant cell joins up with the existing cell wall. Both situations require remodeling of the walls to allow the new synthesis and polysaccharide linkages that must be made.

58.4 RHIZOBIAL ENZYMES THAT DEGRADE PLANT CELL WALLS

Several rhizobia have genes that are predicted to encode pectin or cellulose degrading enzymes (Fauvart et al., 2009) although in *Rhizobium leguminosarum* bv. *trifolii*, neither activity was found extracellularly (Mateos et al., 1992). One secreted pectin-degrading enzyme (HrpW) identified in *Rhizobium etli* was found to be induced in bacteria on the root surface and is likely to be secreted via a Type 3 secretion system. However, mutation of the *hrpW* gene did not affect nodule infection (Fauvart et al., 2009). Possible pectin-degrading enzymes may enable rhizobia to access pectin-based components during saprophyic growth or may even allow the rhizobia to become more embedded in roots by promoting some pectin degradation. Critical for such activity would be a mechanism of secretion across both rhizobial membranes, but the predicted polygalacturonase in *R. leguminosarum* bv. *viciae* (Fauvart et al., 2009) was not identified as one of the proteins secreted by *R. leguminosarum* bv. *viciae* (Krehenbrink and Downie, 2008).

There is evidence that a rhizobial cellulase in *R. leguminosarum* bv. *trifolii* may play a role in infection

(Robledo et al., 2008) because an in-frame deletion removing the cellulase gene (*celC2*) blocked clover infection. However, the *celC2* gene is located within a cluster of genes involved in cellulose synthesis and cellulose synthesis in bacteria requires cleavage of the cellulose that is made. Indeed, the CelC2 cellulase is required for the normal processing of the rhizobiallymade cellulose (Robledo et al., 2012; see Chapter 53). So the question arises as to whether the infection defect in the *celC2* mutant is due to its anomalous production of extended (uncleaved) cellulose filaments, because cellulose synthesis in the mutant is retained. There is good evidence that cellulose production can impair rhizobial infection of root hairs (Laus et al., 2005) and that completely blocking expression of the cellulose synthesis operon does not affect nodulation by different biovars of *R. leguminosarum* (Ausmees et al., 2001; Laus et al., 2005; Williams et al., 2008). These observations suggest that the loss of infection by the *celC2* mutant could be due to altered bacterial cellulose synthesis rather than a lack of plant cellulose degradation. Nevertheless, it is evident that over-expression of the CelC2 cellulase can cause degradation of root-hair cell walls (Robledo et al., 2011) and so the role of CelC2 in the degradation of plant cell wall cellulose cannot be ruled out.

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Chapter 59

Dissecting the Roles in Outer and Inner Root Cell Layers of Plant Genes That Control Rhizobial Infection and Nodule Organogenesis

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59.1 INTRODUCTION

Establishment of the Rhizobium–legume symbiosis (RLS) involves both an intracellular infection process whereby rhizobial bacteria enter the host roots from the soil, and a process of cell division in the cortex of roots, termed nodule organogenesis. Rhizobia subsequently fix atmospheric nitrogen in the newly formed root organs called nodules, to the benefit of the host plant. Before these processes occur, there is a molecular dialog between a host plant and its symbiont in the rhizosphere. In this dialog, rhizobia perceive signals secreted from host roots and, in return, produce and secrete signals that are recognized by the plant. These latter signals are lipo-chitooligosaccharide (LCO) molecules called Nod factors (see Chapter 51). Perception of Nod factors by epidermal plant root cells is an essential first step in the symbiotic process, triggering signaling pathways for the establishment of symbiosis. Understanding the developmental pathways involved in the formation of an intracellularly infected new root organ, and how these pathways are activated by Nod factors, is of fundamental and ecological interest. Several legume species have been exploited to study these questions, notably the model plants *Medicago truncatula* and *Lotus japonicus*, together with their corresponding rhizobial symbionts, *Sinorhizobium meliloti* and *Mesorhizobium loti*, respectively. The genetic tools available in these systems have enabled the dissection of both bacterial and plant determinants of nodulation.

59.2 RHIZOBIAL INFECTION AND NODULE ORGANOGENESIS ARE COORDINATED PROCESSES OCCURRING IN DIFFERENT CELL LAYERS OF THE ROOT

Rhizobial mutants that cannot produce Nod factors are completely deficient for both the epidermal infection process and nodule organogenesis in the root cortex (Denarie et al., 1996). However, while rhizobial infection always depends on Nod factor-producing bacteria, the necessity of rhizobia for the induction of nodule organogenesis depends on the plant species (pure Nod factors are active in certain species, while Nod factor-producing rhizobia are required in others). Studies addressing the role of Nod factor structure in these processes have shown that the infection process is generally more reliant on correct Nod factor structures than nodule organogenesis (Ardourel et al. 1994; Oldroyd and Downie, 2008). That is, rhizobial mutants producing slightly modified Nod factors are generally deficient for infection, but are still able to induce nodule organogenesis.

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However, in such cases, the uninfected nodules that are formed are usually relatively small and often disorganized. One study in *M. truncatula* involving a cortical marker gene, *MtENOD20*, observed cortical cell activation without further nodule organogenesis in response to a certain rhizobial Nod factor mutant, but this mutant induced no epidermal root hair deformation, again showing that cortical responses are less stringent than epidermal ones (Vernoud et al., 1999).

Symbiotic plant genes have been identified that control either rhizobial infection and nodule organogenesis, more specifically just the rhizobial infection process, or just nodule organogenesis. This latter type of gene, together with results from the analysis of rhizobial Nod factor mutants, indicates that epidermal and cortical symbiotic programmes can be uncoupled, with the epidermal infection process apparently being more stringent for Nod factor structure than the process of nodule organogenesis in the root cortex. Plant mutants in the first type of gene are completely deficient for the symbiotic interaction with rhizobia and show very limited or no response to pure Nod factors. Plant mutants in genes that intervene specifically either in the rhizobial infection process or in nodule organogenesis still respond to Nod factors, and although they are primarily blocked either in infection or in nodule organogenesis, these blocks have repercussions on other symbiotic processes such that only small nodules or formed with infection-deficient mutants and infection does not proceed very far in nodulation-deficient mutants. Therefore, these data have not only led to the characterization of epidermal and cortical symbiotic programmes, but have also underlined the necessity of tight coordination between these programmes for a successful interaction. Considering that infection and nodule organogenesis occur simultaneously in different cell layers of the root, with infection then progressing into the nodule primordium formed by cortical cell divisions, it is clear that these processes are interdependent and that there must be mechanisms by which the inner and outer cell layers are coordinated (Oldroyd and Downie, 2008). A recent study has highlighted the coordinating role that bacterial infection exerts on the developing nodule, by describing plant mutants in which defective rhizobial infection is correlated with nodules having central rather than peripheral vascular bundles (Guan et al., 2013). In terms of signaling between the inner and outer cell layers of the root in the early steps of nodulation, the fact that nodule primordium formation precedes infection thread formation indicates an early event of signaling from the epidermis to the inner cell layers of the root. Also, gradients of cell differentiation have been described that show additional waves of signaling, both from the epidermis to inner cells of the root, and for an opposite wave of signaling that travels out from the inner to outer cell layers of the root (Timmers et al., 1999). Indeed, a detailed description of cellular changes occurring during the establishment of nodulation showed that an outward gradient of cell differentiation starts in the pericycle and leads to the formation of a nodule primordium extending from the inner to the middle cortex, while an inward gradient of cell differentiation starts in the outer cortex and results in infection-related cytoskeleton changes that guide the progression of an infection thread into the nodule primordium (Timmers et al., 1999).

59.3 THE NOD FACTOR SIGNALING PATHWAY

The analysis of plant responses to pure Nod factors has shown that many processes are activated, and this has led to a characterization of the different responses as a function of Nod factor concentration and Nod factor structure (D'Haeze and Holsters, 2002). In this way, many, but not all, Nod factor responses are induced by very low Nod factor concentrations, and the majority of responses rely on the same Nod factor structural features that control host specificity. Most responses are detected in epidermal cells, but some can be detected in the cortex and pericycle of the root. The plant genes identified from mutants that are completely deficient in the RLS and show very little or no response to Nod factors, control a Nod factor signaling pathway (Catoira et al., 2000). This pathway is activated via Nod factor perception by plasma membrane-localized receptor proteins, and downstream components of signal transduction, which are largely nuclear components of the cell, are then responsible for the regulation of symbiotic gene expression (Oldroyd, 2013) (Fig. 59.1). Plant genes of the Nod factor signaling pathway are interesting genes for the question of coordination, as they control infection and nodule organogenesis. Moreover, Nod factor signaling appears to occur throughout the process of rhizobial root colonization, not just at the first stages. Studies on these genes by various approaches have provided insights into their roles at different steps of the symbiotic process and in different cell layers of the root.

The Nod factor perception gene, *MtNFP*, is genetically defined as the first gene in the Nod factor signaling pathway in *M. truncatula* (Ben Amor et al., 2003). Models predict that the MtNFP protein is a Nod factor receptor and this is supported by the LysM receptor-like kinase (LysM-RLK) structure predicted for the protein (Arrighi et al., 2006). In *L. japonicus*, the ortholog of MtNFP is called LjNFR5 (Madsen et al., 2003). Nod factor binding was recently reported for LjNFR5, as well as for another symbiotic LysM-RLK protein in *L. japonicus*, called LjNFR1 (Broghammer et al., 2012). Whether MtNFP binds Nod factors is still an open question because several Nod factor-binding sites have been characterized in *M. truncatula* that are independent of MtNFP (Hogg et al., 2006; see Chapter 51). Also, a recently reported role of MtNFP in plant immunity suggests that MtNFP could be a common component of different receptor complexes

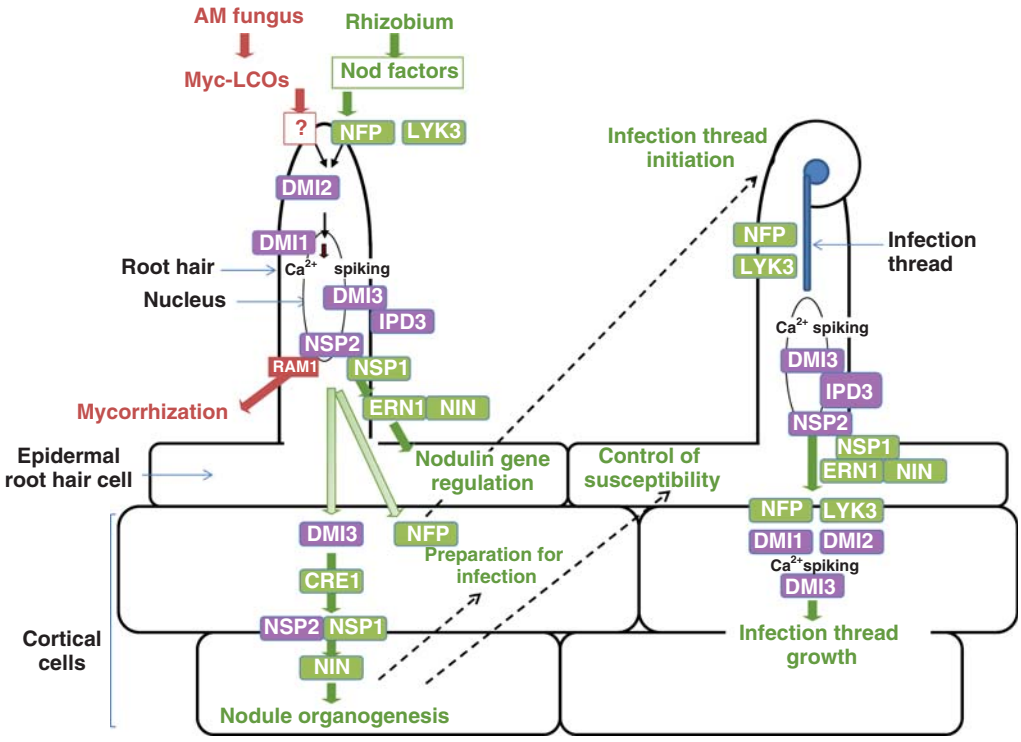

Figure 59.1 Model of early steps of symbiotic signaling in the Rhizobium-legume symbiosis, showing how key components of the plant Nod factor signaling pathway are likely to control nodule organogenesis in the root cortex and infection thread formation in epidermal root hair cells, in response to the perception of Nod factors produced by a rhizobial symbiont. Also shown are the first steps of signaling induced by arbuscular mycorrhizal signals called Myc-LCOs, which involves an as yet unknown Myc-LCO receptor (?), components of the common symbiotic signaling pathway (CSSP) (proteins in purple boxes), and the transcriptional regulator RAM1. Mycorrhizal-specific signaling is depicted in red, while nodulation-specific signaling is depicted in green. Only the *M. truncatula* names of symbiotic plant proteins are given for simplicity. For the activation of nodule organogenesis, Nod factor signaling in a root hair cell generates a secondary signaling mechanism whose interpretation in the root cortex requires DMI3, cytokinin signaling involving the cytokinin receptor CRE1, and the transcriptional regulators NSP1, NSP2 and NIN. For the infection process, the activation of the Nod factor signaling pathway in root hairs leads to the activation of NFP in underlying cortical cells. Cortical NFP, in a Nod factor and DMI3-independent manner, would then signal back to the root hair to control infection thread initiation (dashed line). Infection thread progression in the root hair involves NFP, LYK3, calcium spiking and DMI3, and, subsequently, in response to cortical calcium spiking, cortical DMI3 would be activated to control the progression of the infection thread into the cortex. Bacteria are depicted in blue inside the infection thread. Many other plant genes implicated in the rhizobial infection process are missing from this model. The coordination of epidermal and cortical responses involves as yet unknown mechanisms, but potentially there is signaling from activated cortical cells to control both the susceptibility of the epidermis for infection and the preparation for infection in outer cortical cells (dashed lines).

(Ben et al., 2013; Gough and Jacquet, 2013; Rey et al., 2013). Nonetheless, whatever the molecular function of MtNFP, this protein has a key symbiotic role and *MtNFP* is the only symbiotic gene in *M. truncatula* in which mutants are completely deficient for all symbiotic and Nod factor responses. Furthermore, in *L. japonicus* it was shown that LjNFR5 and LjNFR1 control host specificity, as their transfer into *M. truncatula* enables nodulation by the *L. japonicus* symbiont *M. loti* (Radutoiu et al., 2007).

Downstream of *MtNFP/LjNFR5* is a gene called *MtDMI2* in *M. truncatula* and *LjSYMRK* in *L. japonicus*. The *MtDMI2/LjSYMRK* gene encodes an LRR-RLK, which is a plasma-membrane localised protein, like MtNFP/LjNFR5 (Endre et al., 2002; Stracke et al., 2002). This, together with genetic models, predicts a very early role for MtDMI2/LjSYMRK in Nod factor signaling, although the mechanisms for this are unclear. The Nod factor signaling proteins MtDMI1/LjCASTOR/LjPOLLUX are localized in plant cell nuclei and are involved in the generation of a calcium signal that is induced by Nod factors and is called calcium spiking (Ane et al., 2004; Charpentier et al., 2008; Riely et al., 2007; Venkateshwaran et al., 2012; see Chapter 54). Downstream of *MtDMI1/LjCASTOR/LjPOLLUX* is a gene called *MtDMI3* in *M. truncatula* and *LjCCaMK* in *L. japonicus*. This gene encodes a calcium and calmodulin-dependent kinase that is also localized in plant cell nuclei (Levy et al., 2004; Mitra et al., 2004; Oldroyd and Downie, 2006). A clear role for MtDMI3/LjCCaMK is predicted in the deciphering of the calcium spiking signal, in which the MtIPD3/LjCYCLOPS protein, which is an

interactor of MtDMI3/LjCCaMK (Messinese et al., 2007; Yano et al., 2009), is also implicated.

MtDMI1/LjCASTOR/LjPOLLUX, MtDMI2/LjSYMRK, MtDMI3/LjCCaMK and *MtIPD3/LjCYCLOPS* all control both the RLS and the arbuscular mycorrhizal symbiosis (AMS). As such these genes are part of a common symbiotic signaling pathway (CSSP; also known as CSP or SYM) that is activated in the case of the AMS by mycorrhizal signals called Myc-LCOs, equivalent to Nod factors in the RLS (Fig. 59.1) (Maill et al., 2011; Gough and Cullimore, 2011; Oldroyd, 2013; see Chapters 42, 54, 55, 110). These dual roles of CSSP genes give them a double interest, and to some extent there are similar questions to be addressed in the AMS about signaling between cell layers of the root. *MtDMI3/LjCCaMK* is also particularly interesting as it has been questioned whether MtDMI3 could act as a switch between the CSSP and downstream pathways specific for nodulation and mycorrhization (Godfroy et al., 2006).

The first known components of these downstream pathways are transcriptional regulators. The *NSP1* gene controls a nodulation-specific signaling pathway, while *RAM1* controls a mycorrhization-specific signaling pathway (Gobbato et al., 2012; Heckmann et al., 2006; Smit et al., 2005) (Fig. 59.1). The *NSP2* gene (Kalo et al., 2005; Murakami et al., 2006) was originally thought to act in the same pathway as *NSP1*, but has recently been positioned upstream, as part of the CSSP (Maillet et al., 2011). Both NSP1 and RAM1 interact with NSP2, and they control the transcriptional regulation of nodulation and mycorrhization genes, respectively (Gobbato et al., 2012; Hirsch et al., 2009). Two nodulation-specific genes whose transcriptional regulation is controlled by the NSP1/NSP2 complex are NIN and ERN1 (Andriankaja et al., 2007; Cerri et al., 2012; Hirsch et al., 2009; Marsh et al., 2007; Middleton et al., 2007; Schauser et al., 1999; see Chapter 62) (Fig. 59.1). These are both transcription factors responsible in turn for controlling nodulation-specific signaling downstream of the CSSP (Fig. 59.1). Early "nodulin" genes are among the best characterized genes whose regulation is controlled by the Nod factor signaling pathway in epidermal root hair cells.

This chapter will concentrate on the roles that Nod factor signaling genes play in the RLS, particularly because of the question of coordinating infection with cortical cell divisions, which are absent in the AMS.

59.4 HOW THE EPIDERMAL INFECTION PROCESS IS CONTROLLED BY NOD FACTOR SIGNALING GENES

The strong phenotype of *Mtnfp* mutant plants indicates that MtNFP controls the first step of Nod factor perception, but does not exclude the fact that MtNFP could also intervene in any subsequent steps during the establishment of a symbiotic interaction. Evidence for one or more subsequent roles was provided by a study of the spatio-temporal expression pattern of *MtNFP*. As a potential Nod factor receptor, it was expected that *MtNFP* should be expressed constitutively in epidermal root cells, but interestingly this was not the only profile of expression that was found; *MtNFP* was shown to be expressed throughout the symbiotic interaction with a particularly strong expression in association with the infection process and in cortical cells (Arrighi et al., 2006). This expression pattern inspired studies aimed at deciphering what additional roles MtNFP could play, and whether MtNFP could have distinct epidermal and cortical roles. Firstly, an RNAi approach generated *M. truncatula* roots in which the level of expression of *MtNFP* was reduced. Some of these roots showed the first steps of infection in epidermal root hair cells, but these infections aborted (Arrighi et al., 2006). This showed that MtNFP controls infection as well as early Nod factor signaling, consistent with the fact that Nod factors are produced by rhizobia during infection. Recently, a novel tissue-specific expression approach has addressed the epidermal and cortical roles of MtNFP. For this, *MtNFP* was expressed in *Mtnfp* plants under the control of an epidermal or a cortical-specific promoter, and transformed plants were assessed for their abilities to establish the RLS (Rival et al., 2013, 2012). These experiments produced the intriguing result that epidermal expression of *NFP* was not sufficient to restore rhizobial infection in root hairs. This suggests that NFP fulfills a cortical function in root hair infection and, as it is not expected that Nod factors will reach cortical cells without the presence of rhizobia in infection threads, this cortical role apparently does not involve direct Nod factor perception.

Following the finding that, like *MtNFP*, the *MtDMI3* gene is expressed in both epidermal and cortical *M. truncatula* root cells, a similar tissue-specific expression approach was used with *MtDMI3* (Rival et al., 2013, 2012). In this case, epidermal expression was sufficient to restore rhizobial infection in epidermal root hairs of an *Mtdmi3* mutant, indicating a cell-autonomous role for *MtDMI3* in root hair infection. This is consistent with the induction of calcium spiking in root hair cells. The fact, that the root hair infection threads did not progress into the cortex in plants expressing *MtDMI3* only in the epidermis, is consistent with the observation that calcium spiking is also induced in cortical cells during infection of wildtype plants (Sieberer et al., 2012), and we can hypothesize that MtDMI3 has to be present in the cortex to decipher this calcium spiking. Moreover, a particular frequency of spiking is detected prior to infection of a cortical cell (Sieberer et al., 2012), indicating a role for cortical MtDMI3 in the process of preparation for infection. Other studies on MtDMI3/LjCCaMK have shown the importance of particular domains of the protein for the infection process. Thus, both the truncated

and mutated forms of the MtDMI3/LjCCaMK protein can confer on *MtDMI3/LjCCaMK* mutant plants the ability to form nodules in the absence of rhizobia (Gleason et al., 2006; Tirichine et al., 2007a), but constitutively active forms of the protein that do not carry the regulatory domains can only restore nodulation and not rhizobial infection to *MtDMI3/LjCCaMK* mutants (Takeda et al., 2012). Taken together, activation of the Nod factor signaling pathway in root hairs is necessary, but not sufficient for infection thread initiation. In addition, the underlying cortical cells are apparently somehow involved with an indispensable role of MtNFP (Fig. 59.1).

The epidermal and cortical roles of *MtDMI2/LjSYMRK* are less well characterized, but the expression profile of *MtDMI2* includes epidermal and cortical cells, as well as young nodules and the pre-infection zone of the nodule (Bersoult et al., 2005). Also, the MtDMI2 protein is detected in the plant membrane surrounding infection threads (Limpens et al., 2005). This suggests that MtDMI2 intervenes throughout the symbiotic process, notably in association with infection. The identification of an interesting *LjSYMRK* mutant allele has underlined the important role of this gene during the early steps of infection. The mutation was localized to the extracellular part of the protein, just before the LRR region in a conserved GDPC sequence (Kosuta et al., 2011). Plants carrying this mutation are defective for rhizobial infection of root hairs, but, interestingly, are still able to form nodules, and this latter ability was correlated with low calcium spiking activity (Kosuta et al., 2011). Considering that MtDMI2/LjSYMRK is implicated in generating the calcium spiking signal (Hayashi et al., 2010), and calcium spiking is associated with cortical cells during infection (Sieberer et al., 2012), LjSYMRK/MtDMI2 probably controls both root hair infection and the progression of infection threads across cortical cells. The role of MtDMI2 in nodule infection has also been studied, using an RNAi approach (Limpens et al., 2005). Here, it was shown that MtDMI2 is involved in the formation of symbiosomes, which are organelle-like elements formed by the release of rhizobia from infection threads into the host cells of the nodule. A similar block in symbiosome formation was observed in *DMI2* knock-down lines of the tropical legume *Sesbania rostrata* (Capoen et al., 2005).

Another important genetic component of the infection pathway in *M. truncatula* is the *MtLYK3* gene, which encodes a LysM-RLK very similar to LjNFR1. *Mtlyk3* mutants show normal responses to pure Nod factors, unlike *Ljnfr1* mutants that show practically the same absence of response phenotype to Nod factors as *Ljnfr5* and *Mtnfp* mutants (Radutoiu et al., 2003). This indicates that in *L. japonicus* both LjNFR5 and LjNFR1 control the Nod factor signaling pathway, while in *M. truncatula* MtLYK3 either has no role or a redundant role in Nod factor signaling (Fig. 59.1). Strong *Mtlyk3* mutants show no infection thread initiation, while a weak *Mtlyk3* allele and RNAi plants show some aborted and abnormal infection threads (Catoira et al., 2001; Limpens et al., 2003; Smit et al., 2007). This indicates that wildtype levels of MtLYK3 are needed for normal infection thread formation. Interestingly, these infection defects are more pronounced with rhizobia producing Nod factors with modifications to their structure, indicating a role for LYK3 in recognizing Nod factor structure and making LYK3 a good candidate for a Nod factor receptor controlling the infection process (Limpens et al., 2003; Smit et al., 2007). *Mtlyk3* mutants also show polarity defects in outer cortical cells that are normally associated with the preparation for infection (Catoira et al., 2001), raising the question of whether the function of MtLYK3 to prepare outer cortical cells for infection contributes to the absence of infection threads in mutants. Before rhizobial infection, the LYK3 protein is detected in mobile *puncta* on the plasma membrane of root hair cells (Haney et al., 2011). After rhizobial inoculation these *puncta* become stable, in association with a co-localization of MtLYK3 with flotillin-like 4 (FLOT4). MtLYK3 has also been shown to interact with a remorin protein, MtREM1 (Lefebvre et al., 2010), and as FLOT4 and MtREM1 both have roles in rhizobial infection and are both associated with lipid raft microdomains of membranes, it seems that the recruitment of MtLYK3 to lipid rafts could be important for rhizobial infection. How this could lead to the induction of polarity changes in underlying cortical cells remains to be elucidated.

59.5 HOW THE CORTICAL PROGRAMME OF NODULE ORGANOGENESIS IS CONTROLLED BY NOD FACTOR SIGNALING GENES

Although it has been known for a long time that a functional *MtNFP* gene is needed for nodule organogenesis (Ben Amor et al., 2003), the mechanism for this has only recently been studied. Using tissue-specific promoters, it was shown that epidermal expression of *MtNFP* was sufficient for the activation of cortical cell divisions leading to nodule primordia formation (Rival et al., 2012). This suggests that one function of epidermal NFP is a non-cell autonomous function to generate a secondary signal whose perception is NFP-independent in the root cortex for nodule organogenesis (Fig. 59.1).

Concerning MtDMI3/LjCCaMK, the demonstration that constitutively active forms of the CCaMK can induce spontaneous nodulation in the absence of rhizobia in both *M. truncatula* and *L. japonicus* (Gleason et al., 2006; Tirichine et al., 2007a), demonstrates an important role of MtDMI3/LjCCaMK in nodule organogenesis (see Chapter 54). This spontaneous nodulation is independent of upstream Nod factor signaling genes, including *MtDMI1/LjCASTOR/LjPOLLUX* and *MtDMI2/LjSYMRK*,

indicating that the main role of these components is the activation of the CCaMK (Hayashi et al., 2010; Madsen et al., 2010). This spontaneous nodulation is, however, dependent on downstream components, including NIN (Marsh et al., 2007). A key role of cytokinin in nodule organogenesis was also shown by the ability of constitutively active forms of the cytokinin receptor MtCRE1/LjLHK1 to induce spontaneous nodulation in legume plants in the absence of rhizobia (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Tirichine et al., 2007b). Both NIN and NSP2 are required for the spontaneous nodule organogenesis activated by gain-of-function mutations in MtCRE1/LjLHK1 (Madsen et al., 2010; Tirichine et al., 2007b), while MtCRE1/LjLHK1 is required for spontaneous nodulation with gain-of-function CCaMK (Hayashi et al., 2010). More recently, it was shown that the exogenous application of cytokinin can also induce nodule primordia in *L. japonicus* (Heckmann et al., 2011). Cytokinin-induced nodule primordia formation was maintained in several loss-of-function mutants impaired in epidermal responses including *LjNFR5*, *LjSYMRK* and *LjCCaMK* mutants. However, the absence of primordia in *Ljnsp1*, *Ljnsp2*, and *Ljnin* mutants confirmed the requirement for these transcriptional regulators in the cytokinin-mediated activation of the root cortex. The epidermal and cortical responses were distinguished further when it was shown that external cytokinin application induced the expression of the *NIN* gene within the root cortex, but not in the root epidermis (Heckmann et al., 2011; see also Chapter 56). This, together with other data, positions the cytokinin receptor MtCRE1/LjLHK1 after CCaMK in the signal transduction pathway, and positions the nodule organogenesis activity of the transcription regulators NSP1, NSP2 and NIN, downstream of the cytokinin signal and in cortical cells (Ariel et al., 2012; Hayashi et al., 2010; Madsen et al., 2010; Plet et al., 2011) (Fig. 59.1).

The role of the CCaMK upstream of cytokinin signaling led many models to predict that the role of MtDMI3 in activating cytokinin signaling was restricted to the epidermis. However, the tissue-specific promoter approach showed that *MtDMI3* has to be expressed both in the epidermis and in the cortex for nodule organogenesis (Rival et al., 2012). Taken together, cortical MtDMI3 intervenes in decoding a secondary signal generated by MtNFP and MtDMI3 in the epidermis (Fig. 59.1). This is followed by the activation of cytokinin signaling in the cortex and then by the intervention of the transcription regulators NSP1, NSP2 and NIN, leading to nodule organogenesis (Fig. 59.1). The role of NIN in nodule organogenesis was supported by recent data that showed that NIN over-expression induced root nodule primordium-like structures that originated from cortical cells in the absence of bacterial symbionts (Soyano et al., 2013). This work also indicates that NSP1 and NSP2 act upstream of NIN for nodule organogenesis, and identified two transcriptional targets of NIN that function to stimulate cell division for nodule organogenesis.

59.6 CONCLUSION

Recent data in model legumes have shed light on the roles played by Nod factor signaling genes in the epidermal and cortical symbiotic programmes involved in the establishment of the RLS. In several cases these roles have been pinpointed to the epidermis or to the cortex, with some genes having different roles depending on the tissue. For example, NSP1 and NSP2 control early steps of Nod factor signaling in the epidermis (leading, for example to epidermal nodulin gene induction), while in the cortex they act downstream of the cytokinin receptor for nodule organogenesis. The NIN protein also has dual roles, being essential in the epidermis for rhizobial infection, and controlling nodule organogenesis in the cortex. In addition, both cell autonomous and non-cell autonomous functions have been identified for certain plant genes. For example, nodule organogenesis in the cortex is controlled non-cell autonomously by epidermal MtNFP, while the epidermal rhizobial infection process requires expression of MtNFP both in the epidermis and in the cortex. In this way, both root tissues are implicated in epidermal and cortical symbiotic programmes.

For the infection process in epidermal root hairs, the roles of MtNFP and MtLYK3 in controlling cortical processes underline the importance of the activation of the cortex prior to the infection process. This activation involves pre-infection thread (PIT) formation characterized by polar cytoskeleton rearrangements (Timmers et al., 1999). While the cortical role of MtNFP requires expression in the cortex of the *MtNFP* gene, this question has yet to be addressed for MtLYK3. Another future challenge is to understand how cortical MtNFP controls the process of infection thread initiation, apparently without direct Nod factor perception. As an infection thread develops in an epidermal root hair cell, calcium spiking is induced in underlying cortical cells, and the frequency of the spikes increases to the same frequency as observed with pure Nod factors just before such cells become penetrated (Sieberer et al., 2012). This, together with the necessity of *MtDMI3* expression in both tissues, underlines the importance of Nod factor signaling throughout rhizobial infection in both the outer and inner cell layers of the root (Fig. 59.1). Furthermore, there are specificities in Nod factor signaling mechanisms induced early by pure Nod factors and those implicated in rhizobial infection, as illustrated recently by the study of the promoter region of an early nodulin gene, *MtENOD11*. In response to pure Nod factors the regulation of this gene is controlled by NSP1, NSP2 and ERN1 and involves a regulatory promoter element called the “NF box”, while in response to rhizobium *MtENOD11* induction is controlled by NSP1 and NSP2,

but independently of ERN1, and via a different regulatory region of the promoter region, called "INF" (Cerri et al., 2012).

Future challenges also include the identification of the signaling mechanism by which epidermal MtNFP communicates with the cortex for nodule organogenesis, and the mechanism by which this signal is intercepted in the cortex. The fact that MtDMI3 is indispensable in the cortex for the interpretation of this signal into nodule organogenesis indicates that calcium signaling will be involved. Clearly, cytokinin signaling is subsequently activated in the cortex, and the activity of the transcription regulators NSP1, NSP2 and NIN then leads to the induction of cortical cell divisions for nodule organogenesis (Fig. 59.1).

In summary, rhizobial Nod factors are first perceived by root epidermal cells, and this results in the activation of signaling mechanisms in epidermal and cortical cells, which leads to the establishment of a successful symbiotic interaction. Interestingly, there are signaling mechanisms activated in the cortex both for cortical cell division leading to nodule organogenesis and for epidermal root hair infection. In this way, both tissues take part in controlling a symbiotic process occurring in the other root tissue, and the mechanisms by which the epidermis and the cortex communicate with other for these shared roles contributes to the coordination of rhizobial infection and nodule organogenesis for a successful symbiotic interaction. Many details are still missing concerning the regulatory mechanisms that limit the area of root susceptibility to Nod factor signaling to ensure, for example, the formation of discrete nodule primordia, and the formation of infection threads uniquely above developing primordia (Fig. 59.1). However, NIN has been implicated in such spatial control and a model was suggested in which Nod factor-induced cytokinin signaling could influence epidermal susceptibility to rhizobial infection by participating in the mechanisms that regulate NIN activity (Frugier et al., 2008). More generally, cytokinin signaling could have a role in infection thread formation, and could therefore play a role in coordinating infection and nodule organogenesis (Murray, 2011; see Chapter 56). Cytokinin signaling is also implicated in the control of nodule numbers by interaction with CLE peptides (Mortier et al., 2012). The mechanisms underlying outer and inner cell layer coordination should be better understood in the future with further use of tissue-specific promoters or other tissue-specific approaches such as laser micro-dissection or fluorescence-associated cell sorting (FACS).

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Chapter 60

The *Medicago truncatula* NIP/LATD Transporter Is Essential for Nodulation and Appropriate Root Architecture

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60.1 INTRODUCTION

Legume plants are able to form two different types of secondary organs on their roots: lateral roots (LRs) and symbiotic nitrogen-fixing root nodules. The latter depend on the presence of microsymbiont partners: bacteria called rhizobia. After exchange of specific molecular signals between the rhizobia and host legume, legume root hairs curl, trapping rhizobia in so-called shepherd's crook structures. The legume cell walls breach, initiating the development of infection threads (ITs), enabling the rhizobia to invade the plant root tissue and travel to newly divided root cortical cells. There, rhizobia become encapsulated in plant membranes into host cells, via an endocytosis-like event, forming symbiosomes that resemble acquired organelles. In indeterminate nodules, a persistent nodule meristem and characteristic nodule zones develop from the nodule primordium (Vasse et al., 1990). Both symbiotic partners undergo developmental changes that culminate in the acquisition of nitrogen-fixing ability by the rhizobia (Jones et al., 2007; Oldroyd et al., 2011; see also Chapter 59).

Similarly to nodules, LRs develop adjacent to xylem poles and are initiated as primordia, developing recognizable meristems at their distal ends (Laskowski et al., 1995; Malamy and Benfey, 1997; Malamy, 2005). Because of these shared features, it has been speculated that nodules originated evolutionarily from modified LRs (Hirsch and LaRue, 1997; Mathesius et al., 2000; Szczyglowski and Amyot, 2003), although there are significant differences in the anatomical features of the two types of organs. Despite these differences, a number of legume mutants have been identified that have defects involving both nodulation and LR development, lending support for this idea (Penmetsa et al., 2003; Gonzalez-Rizzo et al., 2006; Couzigou et al.,

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2012). Among these are the *Medicago truncatula nip* and *latd* mutants (Veereshlingam et al., 2004; Bright et al., 2005; Teillet et al., 2008), encoding the MtNIP/LATD (NUMEROUS INFECTIONS AND POLYPHENOLICS/LATERAL ROOT-ORGAN DEFECTIVE) transporter (Yendrek et al., 2010; Bagchi et al., 2012).

Nitrate is both a nitrogen source and a signal that modulates root system development. Nitrate affects root architecture differently if it is applied locally or systemically. High nitrate concentrations supplied locally stimulate LR elongation; in contrast, high nitrate supplied systemically has an inhibitory effect on LR development which increases with nitrate concentration and is especially apparent at high (over 10 mM) nitrate concentrations (Zhang et al., 2000, 2007; Remans et al., 2006; Tsay et al., 2011). While nitrate at concentrations ≥ 1 mM suppresses both incipient nodule development and nitrogen fixation in established nodules (Harper and Gibson, 1984; Streeter, 1988; Fei and Vessey, 2008), there have been suggestions that it may have a different function at lower concentrations (Horchani et al., 2011; Meilhoc et al., 2011). One potential role is as a nitrogen source. The rapidly dividing rhizobia and plant cells in nodule (and LR) primordia constitute nitrogen sinks because of the need for protein and nucleic acid biosynthesis. A second role for the nitrate is as a precursor for nodule nitric oxide (del Giudice et al., 2011; Meilhoc et al., 2011; Cam et al., 2012; see Chapter 64). A third possible role for nitrate is as a participant in an alternative nitrate–nitric oxide respiration pathway recently proposed to be operating in the hypoxic nodule environment (Horchani et al., 2011; Meilhoc et al., 2011). Further, nitrate reductase activity has been linked to nitrogen fixation activity in functional nodules (Cheniae and Evans, 1960) and it could operate in this pathway (Horchani et al., 2011). Thus, nitrate has effects on both LRs and nodules.

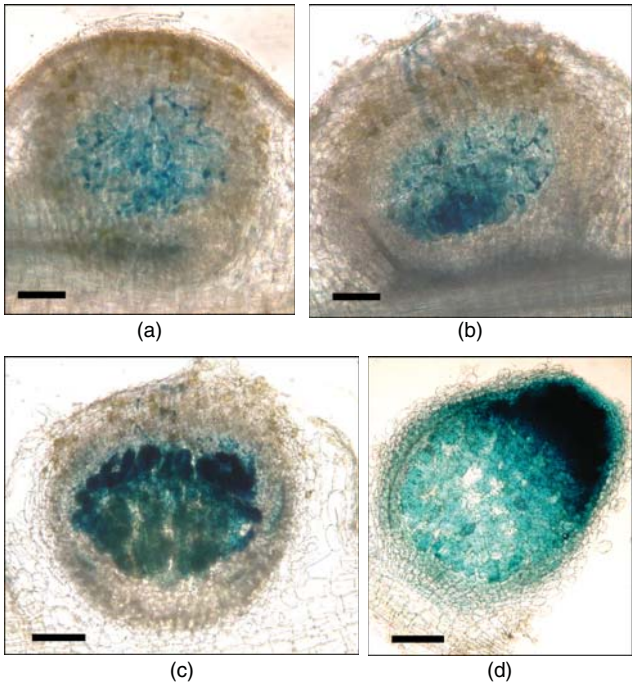

Figure 60.1 Phenotypes of *Mtnip* and *Mtlatd* nodules. *M. truncatula* plants were nodulated with *S. meliloti* containing a constitutive *lacZ* reporter gene. At 15 days post-inoculation, nodule characteristics of mutants were compared to wild-type. The localization of rhizobia was shown by X-Gal staining for the *lacZ*-containing rhizobia, resulting in a blue color. (a) *Mtnip-1*, (b) *Mtlatd*, (c) *Mtnip-3*, (d) Wild-type A17. Bar = 400 μm.

60.2 *Mtnip/latd* MUTANTS' PHENOTYPES

M. truncatula nip and *latd* mutants have defective nodules and root architecture (Haynes et al., 2004; Veereshlingam et al., 2004; Bright et al., 2005; Teillet et al., 2008). When grown without sufficient nitrogen (N), the *Mtnip-1* mutant showed N-deficiency symptoms, even in the presence of *Sinorhizobium meliloti*, *M. truncatula*'s symbiotic partner. Examination of *Mtnip-1*'s small brown bump-like nodules by conventional light-, confocal- and electron microscopy demonstrated that *Mtnip-1* mutants initiated symbiotic interactions, forming nodule primordia containing abnormally proliferative ITs that ultimately failed to deposit rhizobia within host cells (Fig. 60.1a). The ITs in the *Mtnip-1* mutant nodules were observed to be swollen compared to normal ITs in wild-type controls (Haynes et al., 2004; Veereshlingam et al., 2004). It is important to note that even the primary ITs that facilitate movement of rhizobia into the host root/developing nodule in *Mtnip-1* were observed to have abnormal morphology, indicating that the defect in *Mtnip-1* nodule ITs affects the entire rhizobial infection process (Veereshlingam et al., 2004). In the rare cases where rhizobial release occurred, neither host cells nor rhizobia appeared to develop further (Veereshlingam et al., 2004). *Mtnip-1* nodules lacked the hallmarks of differentiated tissues: no identifiable nodule meristem, invasion zone or other central nodule zones were observed (Veereshlingam et al., 2004). Histochemical staining showed that nodules accumulated polyphenolics, characteristic of a defense response. In addition, expression analyses of selected *M. truncatula* genes revealed that *Mtnip-1* nodules expressed only a subset of nodule-specific genes, indicating a block in nodule differentiation (Veereshlingam et al., 2004). A more recent study monitored the localization of MtENOD8-SP-GFP (Early NODulin 8's Signal Peptide fused to Green Fluorescent Protein) and found that it trafficks to the vacuole in root cells or to the symbiosome space in infected nodule cells. ENOD8-SP-GFP was found to localize to the vacuole in *Mtnip-1* nodule primordia cells, suggesting that *Mtnip-1* nodule cells retain root-like/nodule primordium cell characteristics (Meckfessel et al., 2012).

Mtnip-1 displays defective LRs and a semi-penetrant defect in primary root length. Complementation testing showed that *Mtnip-1* was allelic to *Mtlatd* (Veereshlingam et al., 2004), allowing a more thorough investigation of the function of this gene.

Mtlatd is the most severely affected of the mutants. Its nodules are similar to those of *Mtnip-1* (Fig. 60.1b) and were found not to express *S. meliloti bacA* and *nex38* genes, indicating that *Mtlatd* nodule primordia contain rhizobia that fail to make the developmental transition from free-living to symbiotic rhizobia. Rhizobia infecting *Mtlatd* primordia also fail to express *nifH* and thus fail to fix nitrogen (Bright et al., 2005).

Defective root architecture has been very well characterized in *Mtlatd*: LRs in *Mtlatd* are initiated, but arrest as short stumps. Primary *Mtlatd* roots appear to be normal at 3 days post-germination, but by 13 days, more than half cease growth and have deformed root tips. By 22 days post-germination, all *Mtlatd* plants were found to have profound primary root tip defects and to have stopped growth (Bright et al., 2005). *Mtlatd* root hair development was also abnormal. Microscopic examination of both primary and LRs revealed highly disorganized root tips that did not contain identifiable meristems. Thus, it was established that *MtNIP/LATD* is essential for the development and/or maintenance of all root meristems: those of the primary root, LRs and nodules (Bright et al., 2005).

Both *Mtnip-1* and *Mtlatd* were shown to have normal responses to ethylene inhibitors and precursors (Veereshlingam et al., 2004; Liang et al., 2007), suggesting that the ethylene response is normal in the mutants. In legumes and nodulating nonlegumes, abscisic acid (ABA), stimulates LR development, which is opposite to its role in root development in other plants (Liang and Harris, 2005). Interestingly, ABA was found to rescue the root architecture defects in *Mtlatd*, but not the nodulation defects (Liang et al., 2007), which has led to the hypothesis that *MtNIP/LATD* has a role in ABA signaling (Liang et al., 2007; Harris and Dickstein, 2010; Boursiac et al., 2013). In this regard, it is important to note that ABA interferes with Nod factor signaling (Ding et al., 2008; see also Chapter 56) which is essential to the earliest steps in nodulation, prior to the block to nodule development found in *Mtnip/latd* mutants.

Mtnip-3 is the weakest allele of the three existing mutants. It has shorter LRs and primary roots than wild-type, but the roots are not as defective as those of *Mtnip-1* and *Mtlatd*. *Mtnip-3* has Fix+/− nodules that were found to fix about 6% of the acetylene as wild-type in the acetylene reduction assay (Teillet et al., 2008). *Mtnip-3* nodules contain obvious meristems and invaded cells with rhizobia in symbiosomes, but also have the brown pigmentation characteristic of *Mtnip/latd* mutants (Fig. 60.1c) (Teillet et al., 2008).

Collectively, the nodule phenotypes found in the *Mtnip/latd* mutants show a correlation between the release of rhizobia into symbiosomes and the formation of a persistent nodule meristem, suggesting that these developmental hallmarks in nodulation are causally linked. The phenotypes also demonstrate that root and nodule meristems share a common genetic determinant (Veereshlingam et al., 2004; Bright et al., 2005; Teillet et al., 2008).

60.3 MtNIP/LATD PROTEIN, A MEMBER OF THE NRT1(PTR) TRANSPORTER FAMILY

MtNIP/LATD was cloned using a map-based approach and found to encode a member of the nitrate transporter 1(peptide transporter) (NRT1(PTR)) family. The protein is predicted to have eleven transmembrane domains, with a long external *N*-terminal domain and a large cytosolic loop between the fifth and sixth transmembrane domains (Fig. 60.2) (Yendrek et al., 2010). *Mtlatd*, the most severe allele, has a nonsense mutation in *MtNIP/LATD*, rendering it a null mutant, while both *Mtnip-1* and *Mtnip-3* have missense mutations. *MtNIP/LATD* was found to be widely expressed in many tissues of *M. truncatula* and to be repressed by auxin and ABA and up-regulated by cytokinin (Harris and Dickstein, 2010; Yendrek et al., 2010). Its expression was unaffected by nitrate, which is different from many other studied nitrate transporters in the NRT1(PTR) family (Tsay et al., 2011). The use of *MtNIP/LATD* promoter-*GFP* fusions established high levels of *MtNIP/LATD* expression in the nodule, primary root and LR meristems and the surrounding cells (Yendrek et al., 2010).

60.4 MtNIP/LATD BIOCHEMICAL FUNCTION(S)

The NRT1(PTR) family is large, comprising 53 members in *Arabidopsis* and at least 80 members in rice (Tsay et al.,

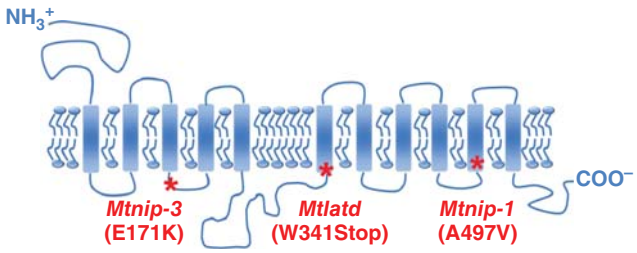

Figure 60.2 The predicted MtNIP/LATD transporter. The protein is predicted to have 11 transmembrane domains, with a long external *N*-terminal domain and a large cytosolic loop between the fifth and sixth transmembrane domains. The positions of the mutations in the *Mtnip/latd* mutants are shown.

2011). Family members that have been functionally studied mediate H^+- dependent transport of nitrate, peptides (Waterworth and Bray, 2006; Tsay et al., 2007), dicarboxylates (Jeong et al., 2004), auxin (Krouk et al., 2010), ABA (Kanno et al., 2012), and glucosinolates (Nour-Eldin et al., 2012). One NRT1(PTR) member, AtNRT1.1, has been shown to serve as a nitrate sensor (Ho et al., 2009). The predicted MtNIP/LATD protein is found in a phylogenetic clade in which the biochemically characterized members are low-affinity nitrate and glucosinolate transporters (Tsay et al., 2007; Yendrek et al., 2010; Nour-Eldin et al., 2012).

Mtnip/latd mutants were investigated for their LR responses when nitrate was provided at low (250 μM) and high (5 mM) concentrations and it was found they were more defective in their responses to low than to high nitrate (Bagchi et al., 2012). However, the mutants displayed no differences in their abilities to take up nitrate from the medium at either concentration (Bagchi et al., 2012).

The *Xenopus laevis* oocyte is a powerful system to functionally assess transporters (Sobczak et al., 2010). Expression of *MtNIP/LATD* in *X. laevis* oocytes demonstrated that it conferred on the oocytes pH-dependent high-affinity nitrate transport with a Km of 160 μM (Bagchi et al., 2012), which is below the concentration at which nitrate becomes inhibitory to nodulation. Mutant *Mtnip/latd* alleles were also tested in the oocyte system: oocytes expressing the more severe mutant *Mtnip-1* and *Mtlatd* alleles were unable to take up nitrate from the medium, but oocytes expressing the less severe *Mtnip-3* allele were not distinguishable in their nitrate transport properties from oocytes expressing the wild-type *MtNIP/LATD* gene (Bagchi et al., 2012). Taken together, these data demonstrate that MtNIP/LATD functions as a high affinity nitrate transporter and suggest that it has another function besides nitrate transport into the plant root.

Functional nitrate transport in plants can be assessed through the use of chlorate. Chlorate is an herbicide that is taken up through the major nitrate transporter, AtNRT1.1(CHL) in *Arabidopsis*, where it is reduced to toxic chlorite that kills the plant (Tsay et al., 1993). *Atchl1-5* mutants contain a deletion that spans the *AtNRT1.1(CHL)* gene and are resistant to chlorate (Munos et al., 2004). Wild-type *MtNIP/LATD*, the strong mutant allele *Mtnip-1*, and the weak allele *Mtnip-3* were each tested by constitutive expression in the *Atchl1-5* mutant to determine if each was able to restore chlorate susceptibility to the *Atchl1-5* mutant. The null mutant allele *Mtlatd*, with a stop codon in the middle of the coding region (Fig. 60.2), was not tested. Results showed that both *MtNIP/LATD* and the weak mutant allele *Mtnip-3*, restored chlorate susceptibility while the strong mutant allele *Mtnip-1* did not (Bagchi et al., 2012; Salehin et al., 2013). These data show that MtNIP/LATD transports the nitrate analog chlorate *in planta*, thus strongly suggesting that MtNIP/LATD transports nitrate *in planta* as well. Because Mtnip-3 is proficient at chlorate transport *in planta*, but the *Mtnip-3* mutant has a nodule phenotype, it also suggests that MtNIP/LATD has another function besides nitrate transport.

Another experimental approach supports the hypothesis that MtNIP/LATD may function as a nitrate transporter *in planta*: *AtNRT1.1(CHL)*, encoding a well-characterized dual-affinity nitrate transporter ((Liu et al., 1999; Liu and Tsay, 2003), was constitutively expressed in *M. truncatula Mtnip-1* mutant roots and found to partially rescue the root architecture phenotype of *Mtnip-1*. However, there was no effect on the nodulation phenotype, while in the controls that constitutively expressed *MtNIP/LATD,* full complementation was observed (Bagchi et al., 2012). These experimental results are confounded by the possibility that *AtNRT1.1(CHL)* mRNA or its product may not be stable in nodules and also by the observation that AtNRT1.1(CHL) transports auxin (Krouk et al., 2010).

Several other possible substrates for MtNIP/LATD have been experimentally tested. *X. laevis* oocytes expressing *MtNIP/LATD* were found to be unaltered in their ability to take up histidine (Bagchi et al., 2012), an amino acid transported by *Brassica napus* and rat NRT1(PTR) proteins (Yamashita et al., 1997; Zhou et al., 1998), as well as several peptide transporters (Tsay et al., 2007). This result suggests that MtNIP/LATD does not transport histidine. Attempted complementation of *Mtnip-1* root systems with *AgDCAT1* cDNA, encoding a dicarboxylate transporter (Jeong et al., 2004) and with *AtNRT2.1* cDNA, encoding a high-affinity nitrate transporter (Kotur et al., 2012; Laugier et al., 2012) did not result in any change in the *Mtnip-1* phenotype (Bagchi et al., 2012; Salehin et al., 2013). While these latter results might suggest that MtNIP/LATD transports neither dicarboxylates nor nitrate at high affinity, they are not conclusive. Indeed, AtNRT2.1 has been recently shown to require AtNAR2.1 for activity (Kotur et al., 2012) and the stability of *AgDCAT1* and its encoded protein in *M. truncatula* are unknown.

60.5 SUMMARY AND PERSPECTIVES

MtNIP/LATD is essential for nodulation and root architecture development. It regulates the development and/or maintenance of the primary root, LR and nodule meristems. It also controls the deposition of rhizobia from ITs into symbiosomes via infection droplets during nodulation and the development of nodule primordia into differentiated nodule cells; it is possible that that these latter blocks to nodule development are consequences of a primary defect in the formation of a nodule meristem. Two mutants, *Mtnip-1* and *Mtlatd*, have severe defects, with *Mtlatd* a null mutant.

The third mutant, *Mtnip-3* has less severe defects (Veereshlingam et al., 2004; Bright et al., 2005; Teillet et al., 2008). *MtNIP/LATD* was cloned and encodes a transporter in the broad-spectrum substrate NRT1(PTR) transporter family (Yendrek et al., 2010) that has a broad spectrum of substrates. It is the first member of the NRT1(PTR)family that has been identified as a high-affinity nitrate transporter and evidence points to its having a second function (Bagchi et al., 2012; Salehin et al., 2013). It has been speculated that MtNIP/LATD might transport a hormone essential for meristem and nodule development (Harris and Dickstein, 2010). Alternatively, it may serve as a nitrate or nitrogen sensor (Bagchi et al., 2012).

What role could MtNIP/LATD transport of low concentrations of nitrate serve? One possibility is that the low nitrate concentration transported may provide bioavailable nitrogen precursors for needed protein and nucleic acid biosynthesis in the dividing cells in meristems and nodules (Bagchi et al., 2012). Another possibility is that the transported nitrate might be a source of nitric oxide. Yet another is that MtNIP/LATD nitrate transport could participate in a nitrate–nitric oxide respiration pathway recently proposed to exist in nodules (Horchani et al., 2011; Meilhoc et al., 2011; Bagchi et al., 2012; see Chapter 64). Future work is aimed at identifying the second function of the MtNIP/LATD protein and understanding its role in nodule and LR development more fully.

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Chapter 61

A MYB Transcription Factor Interacts with NSP2 and Is Involved in Nodulation in *Lotus japonicus*

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61.1 INTRODUCTION

The unique and complex interactions between legume roots and rhizobia lead to the development of a special organ, the root or stem nodule, where rhizobia convert atmospheric dinitrogen into ammonium ion. Symbiotic nitrogen fixation allows the host plants to grow to maturity without the need of exogenous nitrogen fertilizer application. Genetic and genomic studies on nodulation in the model legumes of *Lotus japonicus* and *Medicago truncatula* have identified a set of plant genes essential for the perception and signal transduction of rhizobial Nod factors (Oldroyd et al., 2005; Stacey et al., 2006; see Chapter 59). NFR1 and NFR5 of *L. japonicus* (Madsen et al., 2003; Radutoiu et al., 2003; Radutoiu et al., 2007) and LYK3 and NFP of *M. truncatula* (Amor et al., 2003; Arrighi et al., 2006; Smit et al., 2007; see Chapter 51) are receptor-like kinases that have been shown to serve as the putative receptors for Nod factors. Recent studies have shown that several downstream components of the Nod factor signal transduction pathway also function in the signaling pathway that leads to the establishment of arbuscular mycorrhizal (AM) symbiosis (Kistner and Parniske, 2002; Banba et al., 2008). These protein components form a "common symbiosis pathway" (CSP) (also known as CSSP or SYM pathway; see Chapters 42, 55, 59, 110) that is responsible for relaying the symbiotic signals coming from either the nitrogen-fixing rhizobia or the nutrient-beneficial AM fungi. The shared CSP components include putative ion channels (DMI1/POLLUX and CASTOR) (Ane et al., 2004; Imaizumi-Anraku et al., 2005), LRR Receptor-like kinases (SYMRK/DMI2/NORK) (Endre et al., 2002; Stracke et al., 2002), nucleoporins (NUP133 and NUP85) (Kanamori et al., 2006; Saito et al., 2007), a Ca2+/calmodulin(CaM)-dependent protein kinase (CCaMK/DMI3) (Levy et al., 2004; Mitra et al., 2004; Tirichine et al., 2006), and a nuclear localized coiled-coil protein (CYCLOPS/IPD3) (Messinese et al., 2007; Yano et al., 2008; see Chapters 42, 55, 110).

Following the "CSP", two GRAS family transcriptional regulators NSP1 and NSP2 (nodulation signalling pathway 1 and 2) participate in the nitrogen fixation symbiosis pathway downstream of CCaMK (Kalo et al., 2005; Smit et al., 2005;

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Heckmann et al., 2006; see Chapter 59). Analyses of the NSP1 and NSP2 mutants show that both of them are required for infection thread (IT) growth, Nod factors-induced gene expression, and initiation of nodule primordia, but not for root hair deformation, or calcium influx and spiking (Kalo et al., 2005; Smit et al., 2005; see Chapter 59). Interestingly, NSP2 has recently been shown to promote root colonization by mycorrhizal fungi (Maillet et al., 2011), implicating a role of NSP2 in the AM symbiosis. This suggests that NSP2 does not function exclusively in the nitrogen fixation symbiosis.

GRAS proteins are a family of key plant-specific transcription regulators. They are named after the first three members of the family: GAI (GIBBERELLIC ACID INSENSITIVE), RGA (REPRESSOR of GAI) and SCR (SCARECROW). GRAS proteins play diverse roles in root and shoot development, and in transduction of the gibberellic acid (GA) and phytochrome A signals (Bolle, 2004). The property of intercellular movement through plasmodesmata is a prominent character for GRAS domain proteins (Gallagher and Benfey, 2009). At least 33 and 60 GRAS genes have been identified in *Arabidopsis* and rice, respectively (Tian et al., 2004). Potential orthologs of NSP1 and NSP2 can be found in many nonlegume plant species, including *Arabidopsis* and rice (Kalo et al., 2005; Smit et al., 2005; Heckmann et al., 2006; Murakami et al., 2006). Rice NSP1 and NSP2 are able to fully rescue the nodule symbiosis-defective phenotypes of *L. japonicus nsp1* and *nsp2* mutants (Yokota et al., 2010), indicating that both proteins are functionally conserved in higher plants. Consistent with this, it has recently been found that NSP1 and NSP2 are indispensable for strigolactone (SL) biosynthesis in *M. truncatula* and in rice (Liu et al. 2011).

NIN (nodule inception) is another putative transcriptional regulator that is specific for the root nodule symbiosis. NIN protein shows some similarity to Notch and SREBP (sterol responsive element binding protein) transcription factors in animals. The predicted DNA-binding/dimerization domain of NIN proteins contains a consensus motif conserved in plant proteins and has been implicated in nitrogen-controlled development (Schauser et al., 1999; Marsh et al., 2007). It remains unclear what the exact function of NIN protein is and how it becomes activated. However, the *nin* mutants show unusually excessive root hair curling, abortive infection, and no cortical cell division (CCD) (Schauser et al., 1999; Marsh et al., 2007). *NIN* expression is strongly induced upon inoculation of rhizobia or application of Nod factors, and this induction requires NSP1 and NSP2 (Murakami et al. 2006; Hirsch et al., 2009). *NIN* is also up-regulated by cytokinins and may mediate the crosstalk between Nod-factor and cytokinin signals (Gonzalez-Rizzo et al., 2006; Tirichine et al., 2007; see Chapter 56).

In addition to NSP1, NSP2 and NIN, recent studies have identified more transcriptional regulators required for nodule symbiosis. The *M. truncatula bit1* (*branching infection threads 1*) alleles carry a loss-of-function mutation in the *ERN* (*ERF required for nodulation*) gene, which is identical to *Mt ERN1*, which encodes a transcription factor in the AP2/ERF (APETALA 2/ethylene response element) family that acts downstream of CCaMK-dependent Nod factor signaling (Middleton et al., 2007; see Chapter 62). A parallel study using the yeast one-hybrid screening system shows that three closely related AP2/ERF transcription factors (Mt ERN1, 2, and 3) bind to the Nod factors responsive regulatory unit (the NF box) present in the promoter of Mt *ENOD11* (Andriankaja et al., 2007). Another symbiosis mutant *astray* in *L. japonicus* contains a mutation in Lj Bzf, which is closely related to the *Arabidopsis* HY5, a transcription factor that contains a basic leucine zipper in the *C*-terminus and a RING-finger motif in the *N*-terminus (Nishimura et al., 2002). Moreover, reverse-genetic approaches have led to the functional characterization of additional transcriptional regulators, such as Ms ZPT2-1 (Frugier et al., 2000), Mt HAP2-1 (Combier et al., 2006), Lj ERF1 (Asamizu et al., 2008), and Lj SIP1 (Zhu et al., 2008).

At this stage, it is crucial to understand the transcriptional regulatory networks involved in nodule symbiosis and to identify their target promoters. A recent study shows that Mt NSP1 and Mt NSP2 form a complex that is associated with the promoters of early nodulin genes, such as *Mt ENOD11*, *Mt NIN* and *Mt ERN* (Hirsch et al., 2009). In this study, using the *C*-terminal GRAS domain of Lj NSP2 as bait in yeast two-hybrid (Y2H) screening, we identified a novel MYB family transcription factor named IPN2 (Interacting Protein of NSP2) in *L. japonicus*. The IPN2 protein shows strong transcriptional activation activity and is able to directly bind to the *Lj NIN* promoter in yeast cells and *in vitro*. We also explored the potential role of IPN2 in RN symbiosis.

61.2 METHODS

61.2.1 Y2H Library Screening

For Y2H screenings, bait constructs were made in vector pGBKT7 (Clontech, USA). A cDNA fragment encoding the NSP2 region (105–499 amino acids), which lacks the auto-activation domain (AD), was fused in-frame with the GAL4 DNA-binding domain (BD) in the pGBKT7 vector. Bait constructs were transformed into yeast strain Y187 by the lithium acetate method (Gietz et al., 1995). Screening of interacting clones was carried out via mating according to the manufacturer's instructions (Clontech, USA). A total of 10 million transformants from the cDNA library were selected for growth on the stringent SD/-Leu-Trp-His-Ade drop-out

media. Positive clones were tested for the expression of the *lac*Z reporter via β-galactosidase assays. To validate the observed interactions, prey plasmids were isolated and used for retransformation into yeast AH109. Yeast cells with preys were mated one-on-one in parallel with yeast Y187 cells expressing target baits or the negative control plasmid pGBKT7-Lam (Clontech).

61.2.2 β-Galactosidase Assay

Yeast cells grown in liquid selection media were pelleted and washed twice with Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1.0 mM $MgSO_4$, pH 7.0). The cells were resuspended in 300 µl of Z-buffer and permeabilized by three freeze-thaw cycles in liquid nitrogen and a 37 °C water bath. Cell extracts were added to 0.7 ml of Z-buffer containing 50 mM β-mercaptoethanol and 160 µl of ONPG (*O*-nitrophenyl β-D-galactopyranoside, 4 mg/ml in Z-buffer). After incubation at 30 °C for 30 min or until the yellow colour appeared, the reaction was terminated by addition of 0.4 ml of 1.0 M Na_2CO_3. The reaction mixture was centrifuged for 10 min at 13,000 rpm to remove cell debris. β-Galactosidase activity in the supernatant was measured at 420 nm and expressed in Miller units (Miller, 1972).

61.2.3 Knockdown of *IPN2* By RNAi

For RNAi-1 construction, a 201-bp fragment of the 5′-untranslated region (UTR) with a short coding region of *IPN2* was amplified by PCR with two primer pairs 5′-TCCCCC GGGTCT CTTCTT CAAAAC GCA-3′, 5′- ATCGGA TCCACC TGAGTC TCCTTG AAC-3′, and 5′-AACTGC AGTCTC TTCTTC AAAACG CA-3′, 5′-AACTGC AGACCT GAGTCT CCTTGA AC-3′. For IPN2 RNAi-2 construction, a 250-bp fragment of the 3′-UTR was amplified using 5′-AACTGC AGCCCG GGATGA CTCAAA GCCTGA AGAG-3′ and 5′- ACGCGT CGACGG ATCCAG ATTCCC AACCAA AGAC-3′. The amplification cDNA products were digested with *Sma*I-*Bam*HI and with *Pst*I-*Sal*I, and ligated into the pCAMBIA1301-35S-int-T7 vector, in which the sense and antisense *IPN2* DNA sequence were located in tandem with the Arabidopsis *actin-11* intron between them. This intron-hairpin RNA (ihpRNA) construct was placed behind the CaMV 35S promoter.

The RNAi binary vector was transferred into *Agrobacterium rhizogenes* LBA1334 by electroporation. Plants harbouring transformed hairy roots were transferred to pots filled with vermiculite and sand (1:1) with half-strength Broughton & Dilworth (B&D) medium and grown in a chamber in 16/8 h day/night cycle at 22 °C. After 5 to 7 days, plants were inoculated with *Mesorhizobium loti* MAFF303099 and allowed to continue growing in the same medium.

61.3 RESULTS

61.3.1 Identification of IPN2 as an Interacting Protein of NSP2

In *M. truncatula* and *L. japonicus*, both NSP1 and NSP2 contain the conserved *C*-terminal GRAS domain, but have a variable *N*-terminal region (Kalo et al., 2005; Smit et al., 2005; Heckmann et al., 2006; Murakami et al., 2006). In *M. truncatula*, the fusion of NSP1 and NSP2 to the Gal4 DNA BD leads to auto-activation of the Gal4 Y2H system. Auto-activation is caused by the expression of the variable *N*-terminal regions. Truncated NSP1 (NSP1 156–554) and NSP2 (NSP2 113–508) that lack the *N*-terminal region do not lead to autoactivation in the Y2H system (Hirsch et al., 2009). In an attempt to identify NSP1 and NSP2-binding proteins, we used the GRAS domains as bait to screen a *L. japonicus* root cDNA library (Zhu et al., 2008). Positive interaction clones from the primary screens were tested on stringent selective medium (SD-Trp-Leu-His-Ade). Screening with the GRAS domain of Lj NSP1 did not identify any interacting clone. Screening with the GRAS domain of Lj NSP2 resulted in isolation of two independent positive clones. Sequencing analysis of the two plasmids revealed that the two coding regions represent the same gene encoding a novel protein, designated IPN2 for Interacting Protein of NSP2.

61.3.2 IPN2, a MYB Coiled-Coil Transcription Factor of the GARP Subfamily

The full-length Lj *IPN2* cDNA (GenBank accession no. HQ343457) contains an open reading frame of 1074 nucleotides encoding a peptide of 358 amino acid residues, with a predicted isoelectric point of 8.3 and molecular mass of 40.2 kD. A BLAST search for Lj *IPN2* sequences in the NCBI (National Center for Biotechnology Information) high-throughput genomic sequence database identified a perfect match with a TAC (Transformation-competent Artificial Chromosome) clone (GenBank accession no. AP010214). Comparison between the genomic DNA and the cDNA sequence revealed that Lj *IPN2* is composed of six exons and five introns. Lj IPN2 protein contains a single MYB-like DNA-BD (1R) and a predicted coiled-coil dimerization domain (Fig. 61.1a). MYB transcription factors contain one to three copies (termed R1 to 3) of a highly characteristic DNA-BD. These are typically slightly over 50 amino acids in length. Each domain contains three helices and folds into a helix-turn-helix motif (Fig. 61.1b) with three highly conserved Trp residues regularly spaced by 18 or 19 amino acids, and the motif plays a critical role in stabilizing the DNA BD (Ogata et al., 1992). An alignment of Lj IPN2 1R region, other plant MYB-like DNA-BDs, and the R1, R2 and

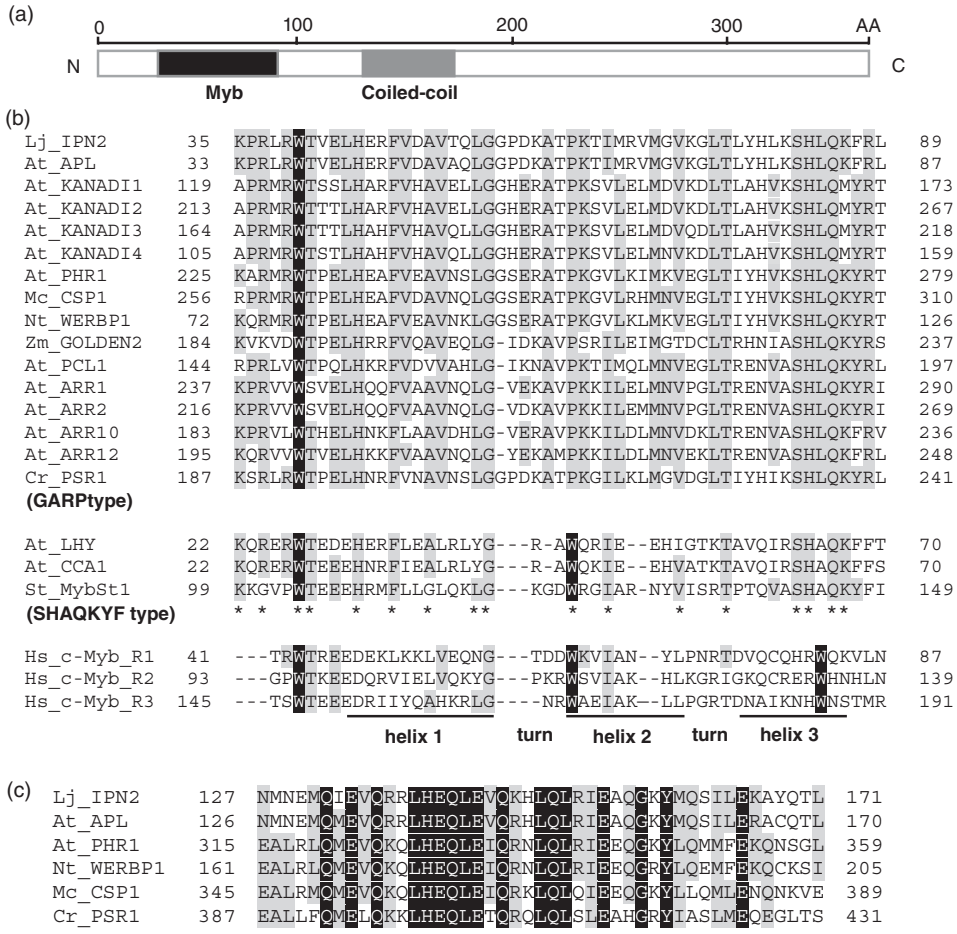

Figure 61.1 IPN2 is a novel plant Myb-related protein belonging to the GARP (GOLDEN2, ARRs, PSR1, and PHR1) subfamily. (a) Schematic illustration of IPN2 protein. Notable features include a single Myb-like DNA binding domain (1R) and a predicted coiled-coil domain. (b) Alignment of the Myb-like domain of IPN2 and the Myb-related domains with the human c-Myb repeats (Majello et al., 1986). The numbers on the left and right indicate the amino acid positions. The three regularly spaced Trp (W) residues are highlighted in reverse contrast. IPN2 has only one conserved 'W' residue and the other two 'W' residues are replaced by 'P' and 'L' in the context of 'SHLQKY/FR', respectively. Thus, IPN2 is classified into the GARP subfamily (Hosoda et al., 2002). (c) Alignment of the predicted coiled-coil conserved domain of IPN2 (amino acids 127–171) with the corresponding regions of other GARP proteins. Identical residues are highlighted in black and conserved residues are shown in grey.

R3 repeats of human c-Myb (Majello et al., 1986) showed that the IPN2 1R region contains only one conserved Trp (W) at the first helix. The other two positions expected to have 'W' residues were replaced with Pro (P) and Leu (L) in the context of 'SHLQKY/FR', respectively (Fig. 61.1b). Based on these substitutions, Lj IPN2 1R region can be classified into the GARP subfamily, which is more distantly related to the MYB superfamily (Riechmann et al., 2000).

Other members in the GARP family include maize GOLDEN2 (G2) (Hall et al., 1998), *Arabidopsis* ARRs (Imamura et al., 1999), *Chlamydomonas reinhardtii* PSR1 (Wykoff et al., 1999), and *Arabidopsis* PHR1 (Rubio et al., 2001). A GARP domain was also found in the recently identified KANADI and APL gene products, which regulates organ polarity and vascular identity in *Arabidopsis*, respectively (Kerstetter et al., 2001; Bonke et al., 2003). The GARP domains are similar to the MYB-like domain of the single repeat MYB proteins CCA1 (Wang et al., 1997), LHY (Schaffer et al., 1998), and MybSt1 (Baranowskij et al., 1994), which, however, have two conserved 'W' residues at the first and second helices and contain the consensus sequence 'SHAQKY/F' in the domain surrounding the third 'W' residue (Fig. 61.1b). Therefore, they are also known as the 'SHAQKY/F' subfamily (Hazen et al., 2005; Fukuzawa et al., 2006).

Besides the GARP domain, Lj IPN2 and some of the GARP family proteins also contain a predicted coiled-coil domain with the consensus sequence 'LHEQLE' (Fig. 61.1c).

61.3.3 Identification of Domains Required for the Interaction Between NSP2 and IPN2

Lj NSP2 contains two leucine heptad repeat domains (LHR1 and LHR2), a VHIID domain, and a Src-homology 2 (SH2)-like domain (Murakami et al., 2006). To determine which domain of NSP2 is responsible for its interaction with IPN2, we constructed the full-length and a series of truncated proteins of NSP2 fused to the Gal4 DNA BD. These constructs were tested for interaction with IPN2 fused to the Gal4 AD (Fig. 61.2a). The strength of interaction was measured using the ß-Galactosidase activity with ONPG as substrate. Most of the *N*- and *C*-terminal deletions of NSP2 failed to interact with IPN2. Only the full-length and a truncated NSP2 (105–499 aa) containing all four domains

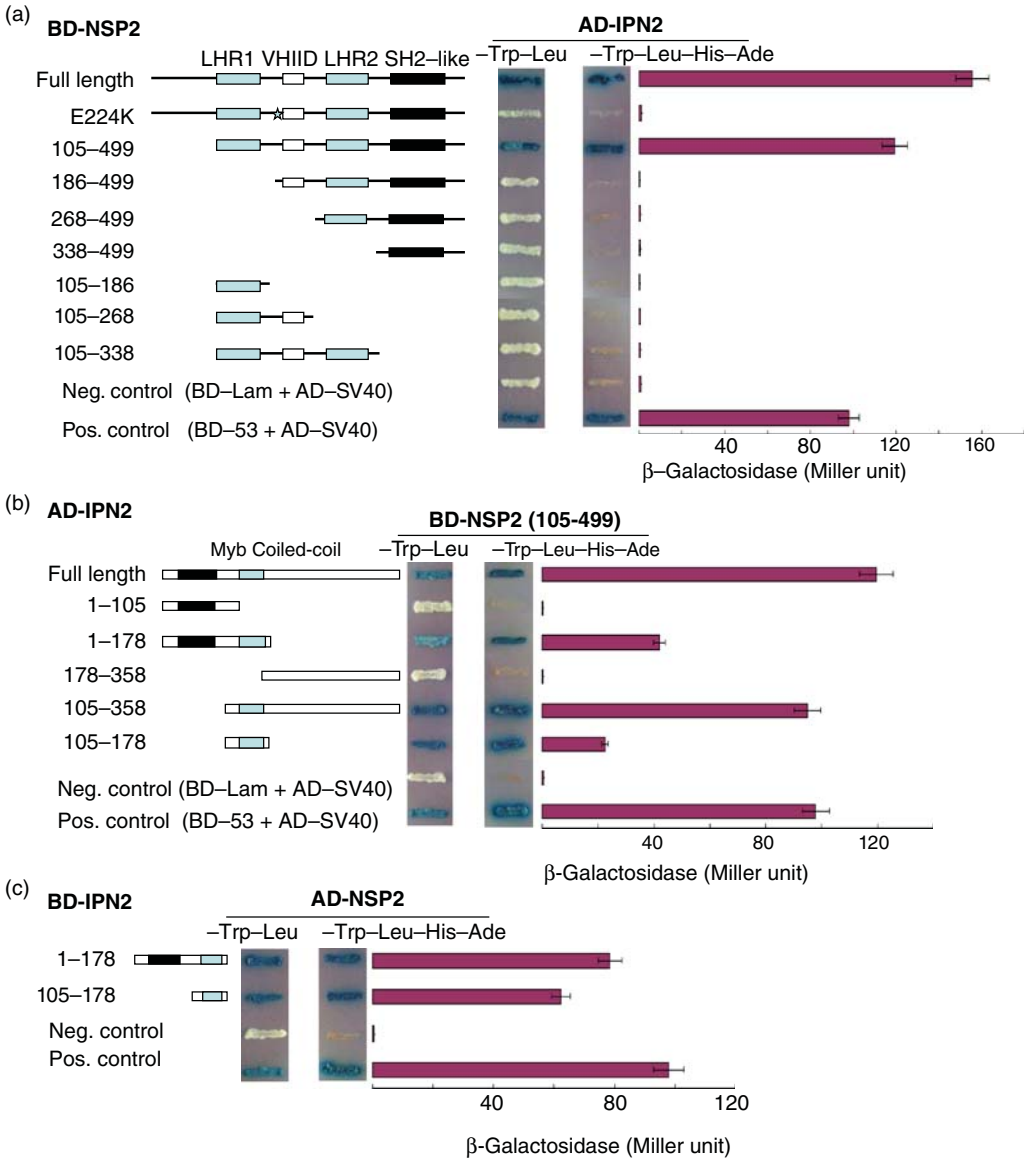

Figure 61.2 IPN2 interacts with NSP2 in yeast. (a) Dissection of the domains of NSP2 required for interaction with IPN2. The *C*-terminal GRAS domain of NSP2 (105–499 aa) was used in the initial isolation of IPN2 from the yeast two-hybrid screening. SD/-Leu-Trp medium was used to test successful mating and SD/-Leu-Trp-His-Ade medium for testing the interaction. The interaction strength was measured through the ß-Galactosidase activity using X-Gal (80 µg/ml) as indicator in plates and ONPG as substrate for quantification. One unit of ß-galactosidase was defined as the amount of enzyme that hydrolysed 1 µmol of ONPG to o-nitrophenol and D-galactose per min (Miller unit). The combination of BD-53/AD-SV40 was used as a positive control and BD-Lam/AD-SV40 as a negative control (Clontech). BD: Gal4 DNA binding domain; AD: Gal4 activation domain. (b) Dissection of the domains of IPN2 required for interaction with NSP2. The coiled-coil domain of IPN2 was necessary and sufficient for interaction with NSP2. (c) Exchanges of Gal4 BD and AD fusion domains. The BD and AD domains of NSP2 and IPN2 in (b) were swapped, and the interaction of the coiled-coil domain of IPN2 with NSP2 was confirmed.

(LHR1, VHIID, LHR2, and SH2-like) were able to interact with IPN2, suggesting that more than one domain of NSP2 is required for this interaction. In contrast, the LHR1 domain of Mt NSP2 alone has been shown to be necessary and sufficient for the interaction with Mt NSP1 (Hirsch et al., 2009). Interestingly, a site mutant (E224K) of Lj NSP2, which is equivalent to the E232K mutant of Mt NSP2, abolished the interaction with Lj IPN2. The Mt $NSP2_{E232K}$ mutant was originally identified in the *nsp2-3* mutant of *M. truncatula*, a weak *nsp2* mutant allele showing limited nodule formation in contrast to the null allele *nsp2-2* showing no nodule formation (Kalo et al., 2005). This mutation also abolishes auto-activation in the Y2H system, and can still interact with Mt NSP1 (Hirsch et al., 2009).

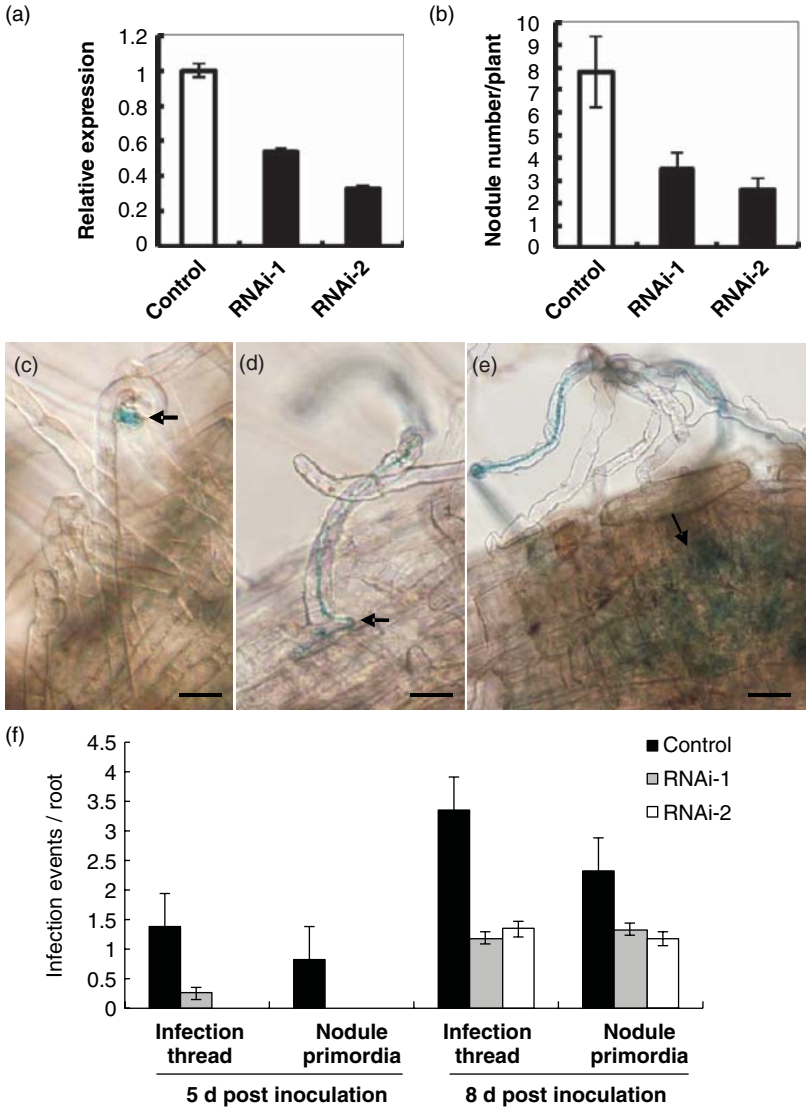

Figure 61.3 Analysis of nodulation phenotypes and rhizobial infection of *IPN2*-RNAi hairy roots. (a) Quantitative RT-PCR analysis of *IPN2* expression levels in the control hairy roots expressing the empty vector (Ct) and representatives of hairy roots expressing the *IPN2*-RNAi constructs (RNAi-1 and RNAi-2). (b) Total nodule numbers of transgenic hairy roots with the vector control and two RNAi constructs were recorded 3 weeks after inoculated with *M. loti* MAFF303099. (c–e) Infection threads (ITs) formation in *IPN2*-RNAi hairy roots. ITs were visualized after staining with X-Gal in hairy roots 8 d post inoculation (DPI) with *M. loti* expressing a *lacZ* construct. The images show the key steps of *Rhizobium* infection in hairy roots, including curled root hairs with entrapped bacterial colony (c), IT growth (d), and release of rhizobia in developing nodule cells (e). Bars = 25 μm for (c) and (d), and 50 μm for (e). (f) Frequencies of the infection events per root of the control hairy roots, *IPN2* RNAi-1 and *IPN2* RNAi-2 hairy roots at 5 DPI and 8 DPI, respectively. The data are presented as 15 individual transgenic plants each construct, and randomly scored in four roots between 4 and 6 cm per plant.

The coiled-coil conformation has been considered as a potential homodimerization or heterodimerization motif (Lupas et al., 1991; Rubio et al., 2001; Parry et al., 2008). To determine whether the coiled-coil domain of Lj IPN2 was responsible for the interaction with Lj NSP2, several deletion mutants of Lj IPN2 fused to Gal4 AD were tested for interaction with the Lj NSP2 (105–499) fused to Gal4 BD (Fig. 61.2b). We found that only the deletion mutants containing the coiled-coil domain interacted with Lj NSP2. On the other hand, the truncated Lj IPN2 (1–178) and Lj IPN2 (105–178) interacted with the full-length Lj NSP2 fused to Gal4 AD (Fig. 61.2c). These results indicate that the coiled-coil domain of Lj IPN2 is necessary and sufficient for interaction with Lj NSP2.

61.3.4 Impairment of Nodulation by *IPN2* RNAi

To elucidate the function of Lj *IPN2* in the nodulation process, we generated transgenic hairy roots expressing *IPN2* RNAi. Transgenic hairy roots expressing the empty vector served as a control. Two *IPN2*-specific RNAi constructs, with *IPN2* RNAi-1 targeting the 201-bp 5′ region and RNAi-2 targeting the 250-bp 3′ region, were expressed in transgenic hairy roots of *L. japonicus*. The levels of the *IPN2* transcripts in transgenic hairy roots were measured by quantitative RT-PCR. The data showed that the *IPN2* transcripts were reduced on average to 51% in *IPN2* RNAi-1 and 32% in RNAi-2 as compared to that in the control hairy roots (Fig. 61.3a).

The hairy roots were inoculated with *M. loti* MAFF303099 and grown for 3 weeks in a nitrogen-free environment. Nodulation phenotypes were examined. The average number of nodules formed on control hairy roots was 7.8, while that on RNAi-1 and RNAi-2 hairy roots was reduced to 3.5 and 2.7, respectively (Fig. 61.3b). This reduction in nodule number by RNAi expression was statistically significantly different from that of the control ($P < 0.01$, $n = 55$ and $P < 0.01$, $n = 61$, respectively). These results indicate that down-regulation of *IPN2* gene expression by RNAi had a negative effect on nodulation.

To determine how *IPN2* RNAi expression affected nodule formation, we analyzed the rhizobial infection process. For this, lacZ-labelled *M. loti* cells (Kang et al., 2011) were used to infect the transgenic RNAi hairy roots, and the presence of ITs were visualized under the microscope. At 5 d post inoculation (DPI), no ITs were observed in the *IPN2* RNAi-2 roots, and occasionally only a few ITs could be observed in the RNAi-1 roots while no nodule primordia could be identified. By contrast, both ITs and nodule primordia could be readily observed in the control hairy roots. The complete absence of ITs at 5 DPI in *IPN2* RNAi roots was surprising, so we recorded ITs at 8 DPI. The initiation of ITs from curled root hair tips (Fig. 61.3c) and ITs growth through well-elongated root hairs to root epidermis (Fig. 61.3d) could be observed in *IPN2* RNAi roots. Also, some ITs could reach to the nodule primordia and rhizobia could be released from ITs into the nodule cells (Fig. 61.3e). Compared to the control hairy roots, the average numbers of ITs and nodule primordia were significantly reduced in *IPN2* RNAi roots (Fig. 61.3f). Thus, both rhizobial infection and nodule organogenesis appeared to be impaired by the knockdown expression of *IPN2* in RNAi hairy roots.

61.4 DISCUSSION

Formation of a transcription factor complex is a key step in order to bind DNA and regulate gene expression. Two GRAS family transcription factors, SCARECROW (SCR) and SHORTROOT (SHR), interact with each other and control root radial patterning (Cui et al., 2007). The *Solanum tuberosum* GRAS protein RGA interacts with the PIF4 basic helix-loop-helix (bHLH) transcription factor, and functions to integrate the light and GA signals (de Lucas et al., 2008).

In *M. truncatula*, GRAS family transcription factors, Mt NSP1 and Mt NSP2 form a complex at the DNA level, and induce specific gene expression changes essential for the root nodule symbiosis (Hirsch et al., 2009; see Chapter 59). In this study, we identified a novel MYB transcription regulator, Lj IPN2, which interacts with Lj NSP2. In *M. truncatula*, the LHR1 domain of Mt NSP2 is necessary and sufficient for interaction with Mt NSP1, whereas the whole GRAS domain of Mt NSP1 (156–554 aa) is required for interaction with Mt NSP2 (Hirsch et al., 2009). In contrast, the whole GRAS domain of Lj NSP2 (105–499 aa) is required for interaction with Lj IPN2, whereas the coiled-coil domain of Lj IPN2 is necessary and sufficient for interaction with Lj NSP2 (Fig. 61.2).

Having an *N*-terminal MYB-like DNA BD, IPN2 belongs to the large MYB protein family. The MYB protein family is universal in the plant kingdom, and MYB domain proteins are classified based on the numbers of MYB-DNA BD repeats. As there is only one MYB domain, Lj IPN2 is classified as a MYB-related protein. The functions of MYB domain family of proteins vary according to the number of repeats as MYB proteins bind to DNA in different ways. MYB proteins with two MYB repeats (R2R3) represent the biggest family in plants. To date, over 100 members have been isolated in *Arabidopsis* and they play various roles in the regulation of secondary metabolism, tissue morphogenesis, phloem formation and cell cycle progression (Riechmann et al., 2000). The Myb-related transcription factors exist widely in plants. They are involved in diverse biological processes including cell proliferation, differentiation and organ development (Stracke et al., 2001; Marian et al., 2003).

Protein domain sequence analysis revealed that the Myb-like DNA BD of IPN2 is closely related to the B motif or GARP motif (Hosoda et al., 2002). The B motif was originally identified as a signature of the type-B ARRs involved in His-Asp phosphorelay systems in *Arabidopsis* (Imamura et al., 1999). The B motif is a representative of the GARP motifs commonly observed in GARP family plant transcription factors, including maize G2 and *C. reinhardtii* PSR1 (Riechmann et al., 2000). G2 controls the differentiation of a photosynthetic cell type of the maize leaves (Hall et al., 1998), whereas PSR1 is a regulator of phosphorus metabolism (Wykoff et al., 1999). *Arabidopsis* PHR1, a protein homologous with PSR1, functions in phosphate starvation signalling (Rubio et al., 2001). The recently identified KANADI gene product, which possesses a GARP motif, has been reported to regulate organ polarity in *Arabidopsis* (Kerstetter et al., 2001). In these cases, the GARP proteins appear to be involved in plant-specific processes. Although their amino acid sequences are distantly related to those of authentic mammalian Myb repeats, little is known about the structure and function of the GARP motif.

Besides the GARP motif, IPN2 also contains a predicted coiled-coil domain annotated as the LHEQLE motif. The combination of the two motifs in one protein peptide is widely found in the plant kingdom from green algae to higher plants (Fig. 61.1b, c). However, the overall structure of IPN2 appear to be unique to higher land plants, as genes coding for proteins with similar structures could not be found in the unicellular algae *C. reinhardtii*, the moss *Physcomitrella patens*, and the lycophyte *Selaginella moellendorfii*. IPN2-like proteins are clearly present in

most of the higher plant species including the dicots and monocots in the databases. Among them, *Arabidopsis* APL is the only one, whose biological function has been characterized. APL is essential for the vascular tissue identity in roots (Bonke et al., 2003). The presence of homologs in nonlegume plant species suggests that IPN2 possibly has a nonsymbiotic function. During evolution, IPN2 may have acquired an additional function in nodulation signalling in legume species.

In summary, we have described the identification of IPN2 as an interacting partner of NSP2 in *L. japonicus*. IPN2 is a MYB coiled-coil type transcription factor belonging to the GARP protein family, while NSP2 is a GRAS family transcription regulator. The two proteins may form a transcription factor complex and promote the expression of a specific set of genes essential for nodule development.

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Chapter 62

AP2/ERF Transcription Factors and Root Nodulation

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62.1 INTRODUCTION

Legumes are capable of intimately associating with rhizobial bacteria in the soil, and this unique symbiosis involves the formation of a specialized root organ, the root nodule, in which the bacteria reduce atmospheric nitrogen to a form that can be assimilated by the host plant. This beneficial interaction is initiated by the specific recognition of bacterial-produced Nod factor (NF) signals that lead to the induction of a unique nodule developmental program, involving rhizobial infection of external root epidermal/outer cortical tissues and nodule organogenesis within the root cortex (see Chapters 51, 59). The coordination of infection and organogenesis occurring in different root tissues leads to the formation of mature nodule structures, in which rhizobia are hosted intracellularly and differentiate into bacteroids which fix nitrogen for the benefit of the host plant (reviewed by Murray, 2011; Oldroyd et al., 2011; Popp and Ott, 2011; see also Chapter 59).

Transcriptomic studies conducted in a number of legume plants have revealed that thousands of genes are expressed during nodule development, including hundreds of Transcription Factor (TF)-encoding genes likely to play central roles in the regulation of this process (Udvardi et al., 2007; Høgslund et al., 2009; Moreau et al., 2011). TFs are represented by diverse gene families which are recognized by the presence of specific DNA binding (DB) domains that interact in a sequence-specific fashion with promoter regions, regulating the expression of their target genes, positively or negatively. Genome-wide analyses of sequenced genomes have revealed that TF gene families are equally distributed between legumes and nonlegume plant species, suggesting that legume-specific traits such as the ability to form nitrogen fixing nodules is not related to TF gene family specialization but rather to differences in TF gene expression patterns and functions (Benedito et al., 2008; Libault et al., 2009, 2010).

TF genes involved in the nodulation process have been identified in legume plants by different strategies, and additional detailed expression and/or functional analyses have made it possible to correlate TF function with specific stages of nodulation (Table 62.1). Firstly, direct genetic screening for nodulation-deficient mutants led to the identification of key TFs essential for both early and late stages of the nodulation process. This includes the GRAS (NSP1 and NSP2), NIN and AP2/ERF (ERN1) transcription factors characterized in *Medicago truncatula*, *Lotus japonicus* and/or *Pisum sativum*. By a complementary yeast one-hybrid screen strategy, ERN1 and two closely related AP2/ERF TFs referred to as ERN2 and ERN3, were identified as important players of early NF signaling, able to bind to the NF-responsive NF-box regulatory sequence as will be discussed later. Genetic strategies based on the identification of supernodulation mutants allowed the identification of the bZIP HY5 TF as a negative regulator of the genetic program leading to nodule development in *L. japonicus* (Table 62.1). Finally, a number of

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Table 62.1 Legume TF-genes characterized during nodulation

Family	Name	Identification Strategy	Nodulation Function	Legumes	References
GRAS	NSP1	Genetics	NF signaling, Infection, Nod org	*Mt, Lj*	Catoira et al., 2000 Oldroyd and Long, 2003 Kaló et al., 2005
	NSP2	Genetics	NF signaling, Infection, Nod org	*Mt, Lj*	Smit et al., 2005 Heckmann et al., 2006 Murakami et al., 2006
NIN	NIN	Genetics	NF signaling, Infection, Nod org	*Mt, Lj, Ps*	Schauser et al., 1999 Borisov et al., 2003 Marsh et al., 2007
AP2/ERF	ERN1	Genetics, yeast 1H	NF signaling, Infection, Nod org	*Mt*	Middleton et al., 2007 Andriankaja et al., 2007 Cerri et al., 2012
	ERN2	Yeast 1H	NF signaling and Infection	*Mt*	Andriankaja et al., 2007 Cerri et al., 2012
	ERN3	Yeast 1H	NF signaling?	*Mt*	Andriankaja et al., 2007
	EFD	Transcriptomics	Nod differentiation	*Mt*	Vernié et al., 2008
	ERF1	Transcriptomics	positive regulator of nodulation	*Lj*	Asamizu et al., 2008
NF-Y	NF-YA1	Transcriptomics	Nod organogenesis	*Mt, Lj*	Combier et al., 2006 Laloum et al., 2012 Soyano et al., 2013
	NF-YB1	Transcriptomics	Nod organogenesis	*Lj*	Soyano et al., 2013
	NF-YC1	Transcriptomics	positive regulator of nodulation	*Pv*	Zanetti et al., 2010
ARID	SIP1	Yeast 2H	SYMRK receptor kinase interactor	*Lj*	Zhu et al., 2008
bHLH	bHLH1	Transcriptomics	Vascular patterning, nutrient exchanges	*Mt*	Godiard et al., 2011
	bHLH476	Transcriptomics	Cytokinin signaling	*Mt*	Ariel et al., 2012
bZIP	ATB2	Transcriptomics	Nod senescence	*Mt*	D'haeseleer et al., 2010
	HY5	Genetics	Nod autoregulation	*Lj*	Nishimura et al., 2002
WUSCHEL	WOX5	Transcriptomics	Nod organogenesis	*Mt, Ps*	Osipova et al., 2012
MYB	CND	Transcriptomics	Nod development	*Gm*	Libault et al., 2009
NAC	NAC969	Transcriptomics	Nod senescence	*Mt*	de Zélicourt et al., 2012

Nod, Nodule; *Mt*, *Medicago truncatula; Lj*, *Lotus japonicus; Ps*, *Pisum sativum*; *Pv*, *Phaseolus vulgaris*; *Gm*, *Glycine max*.

nodule-expressed TFs belonging to different gene families were identified by transcriptomics before being further characterized throughout nodulation (Table 62.1). This review is focused on the AP2/ERF family of TFs, and in particular those related to nodule development in the model legume *M. truncatula*.

62.2 THE AP2/ERF FAMILY IN PLANTS

The AP2/ERF superfamily is one of the largest groups of TFs in plants and is defined by the presence of a conserved DB domain consisting of approximately 60 amino acid residues (for a review see Nakano et al., 2006; Yamasaki et al., 2013). This DB domain was first identified in the Arabidopsis AP2 floral homeotic gene (Jofuku et al., 1994) and in ethylene-inducible DB proteins that interact with an ethylene responsive element (Ohme-Takagi and Shinshi, 1995), giving rise to the name of the gene family. Since then, AP2/ERF or related DB domains were found in a number of plant species but also in non-plant bacterial or viral endonucleases, supporting a potential acquisition of this DB domain by plants following direct gene transfer (Magnani et al., 2004; Shigyo et al., 2006). Interestingly, AP2/ERF-like domains were also found in apicomplexan *Plasmodium* spp., that despite having an overall low homology, exhibit a domain folding similar to the one predicted for the plant AtERF1 (Allen et al., 1998; Painter et al., 2011).

Genome-wide studies have identified AP2/ERF superfamily members in a number of plant species: 157 in Arabidopsis, ∼180 in rice, 126 in *M. truncatula* and 136 in *L. japonicus* (Nakano et al., 2006; Mochida et al., 2010; Zhang et al., 2011). On the basis of amino acid conservation and the number of AP2/ERF domains, this superfamily has been further divided in three major families: the AP2 family which contains proteins with two repeated AP2/ERF DB domains, the RAV (Related to ABI3/VP1) family which

contain proteins with an AP2/ERF and a B3 DB domain and the ERF family with proteins comprising a single AP2/ERF DB domain. While the AP2 members are mainly involved in flower developmental processes, the RAV group is related to hormonal signaling, and the predominant ERF group to a variety of biological processes, and in particular plant responses to environmental conditions as discussed below.

The ERF group comprises the vast majority (>80%) of genes in the AP2/ERF superfamily (e.g., 132/157 in *Arabidopsis*, ~150/180 in rice, 106/126 in *M. truncatula* or 122/136 in *L. japonicus* (Mochida et al., 2010; Zhang et al., 2011) and is further divided into two major subfamilies, termed CBF/DREB (C-repeat binding factor/dehydration responsive element binding factor) and ERF (Fig. 62.1)

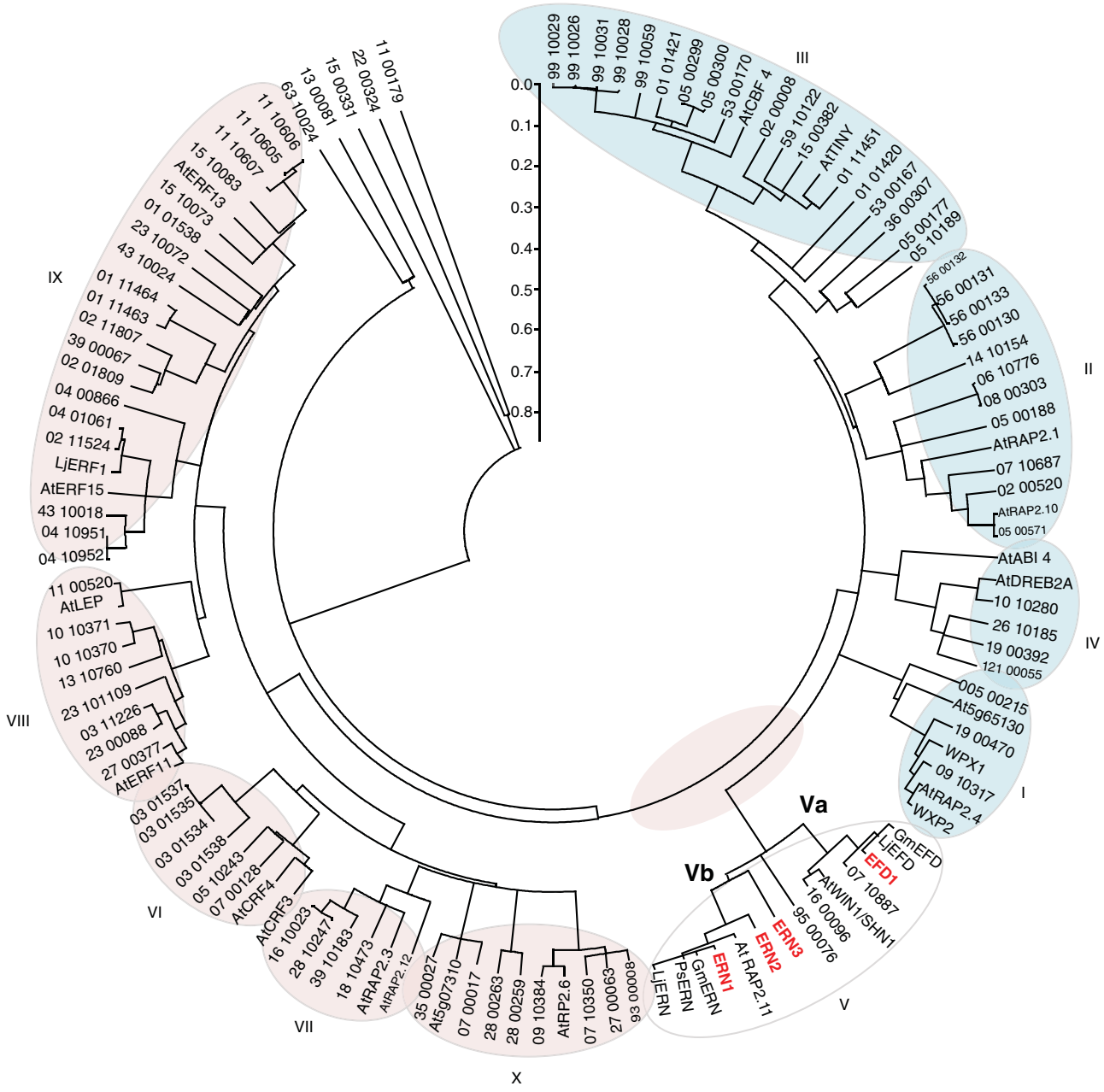

Figure 62.1 Phylogenetic analyses of nodule-expressed *M. truncatula* ERF proteins represented in Table 62.2. A tree of 95 aligned *M. truncatula* sequences was constructed using MEGA 5 (maximum likelihood method and JTT model). The 10 major ERF groups (I to IX) identified by Nakano et al. (2006) classified into respective CBF/DREB (I to IV) or ERF (V to X) families are shaded in blue or pink respectively. EFD1 and ERN TFs are indicated in red. For comparison, *A. thaliana* ERFs, *L. japonicus* ERF1, EFD- and ERN-like sequences from other legumes (Ps, *P. sativum*; Lj, *L. japonicus*; Gm, *G. max)* were included in the tree.

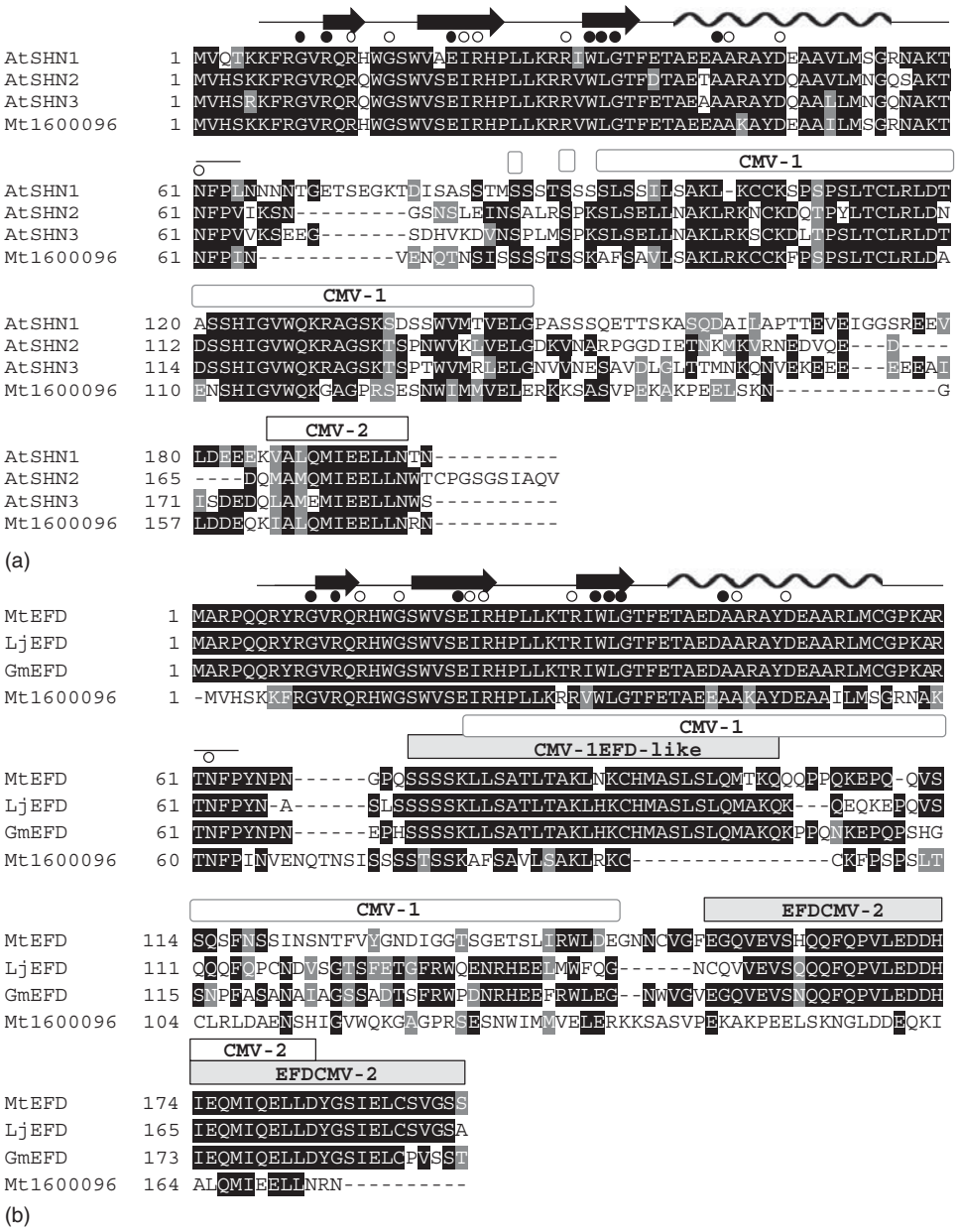

Figure 62.2 Conserved domains in Va ERF TFs in Arabidopsis and legume species. (a) Sequence alignment of the three *Arabidopsis* SHN proteins and their putative ortholog from *M. truncatula* (Fig. 62.1). All four proteins contain a conserved DB domain with 3 β-sheets (arrows) and one α-helix, and two conserved CMV-1 and CMV-2 motifs as indicated by rectangles. (b) Sequence alignment of the three *M. truncatula* group Va ERF TFs, which include the SHN homolog and EFD-like proteins. All four proteins contain a conserved DB domain with 3 β-sheets (arrows) and one α-helix. The positions of CMV-1 and CMV-2 motifs are indicated by open rectangles, while legume conserved motifs are indicated by grey rectangles. Circles indicate the positions of amino acid residues in the AP2/ERF domain being totally (solid circles) or more than 95% (open circles) conserved.

respectively. Taking into account protein conservation throughout the entire ERF protein, Nakano et al. (2006) have classified them into ten major phylogenetic groups with derived sub-groups. Since then, the phylogenetic relationships between members of the ERF group has been studied in a number of other plant species (e.g., rice, poplar or tomato; Nakano et al., 2006; Rashid et al., 2012; Zhuang et al., 2008; Pirrello et al., 2012). This group is characterized by the presence of certain amino acid residues within the AP2/ERF DB domain, either totally or more than 95% conserved as indicated in Figures 62.2 and 62.3 (Nakano et al., 2006). Nevertheless, the overall homology of the entire AP2/ERF DB domain can vary significantly between different ERF phylogenetic groups, from approximately

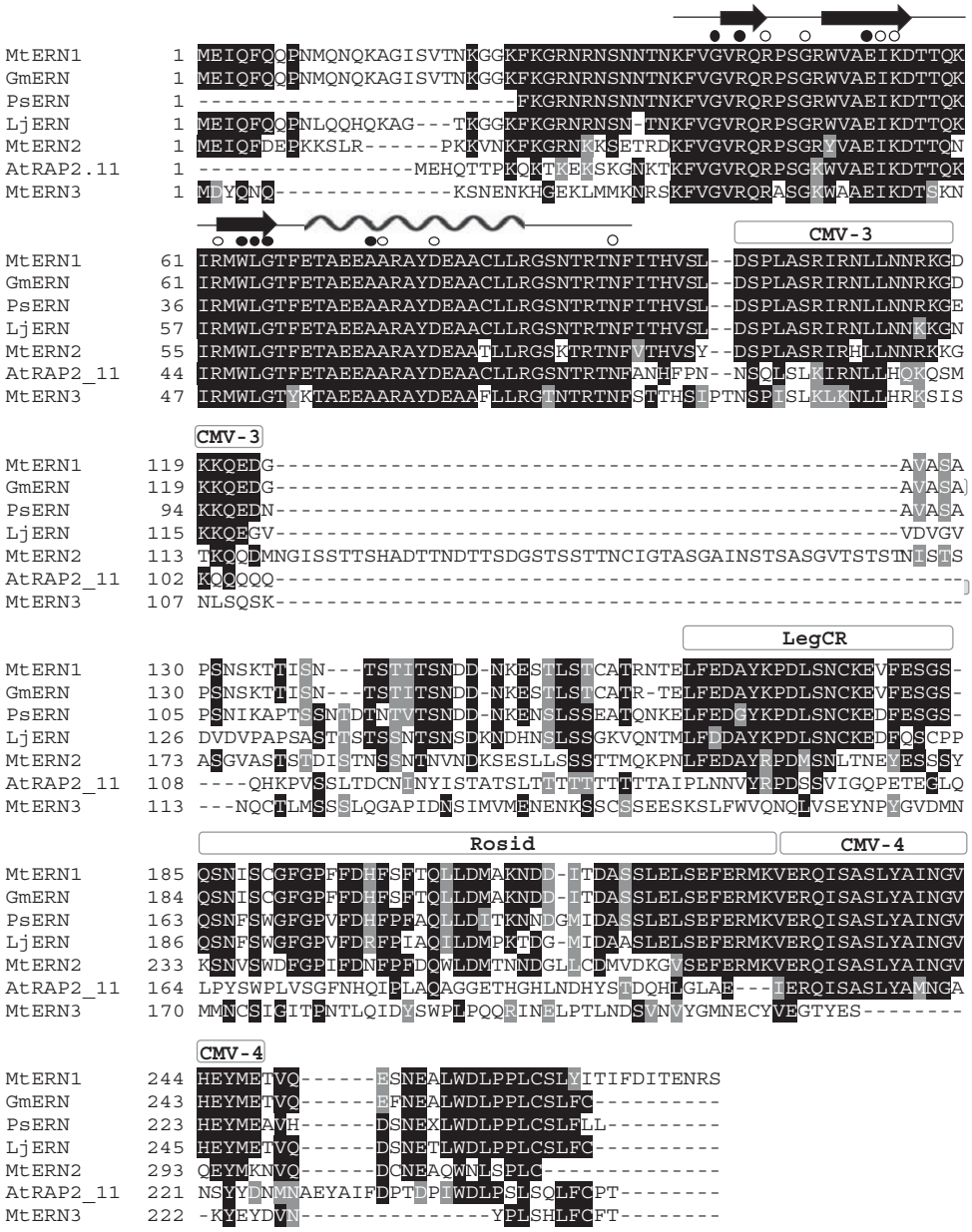

Figure 62.3 Conserved domains in ERN-related TF factors. Sequence alignment of legume (*M. truncatula, L. japonicus, G. max and P. sativum*) ERN-like proteins and the Arabidopsis RAP2.11 ortholog. All four proteins contain a conserved DB domain with 3 β-sheets (arrows) and one α-helix. CMV-3, CMV-4 and the rosid-specific regions are indicated by open rectangles. Circles indicate the positions of amino acid residues in the AP2/ERF domain being totally (solid circles) or more than 95% (open circles) conserved.

50% to 95%. This heterogeneity can be related to different DB specificities as suggested by protein-DB microarray analyses (Gong et al., 2008).

To date, the DB domain of ERF TFs has been shown to recognize GC-rich motifs found in the promoters of regulated genes, including respectively the GCC box (AGCCGCC) of defense-related genes (Ohme-Takagi and Shinshi, 1995), the DRE (dehydration response element) A/GCCGAC element of stress-related genes (reviewed by Mizoi et al., 2012) and the GCC-like motif (GCAGGC) of symbiotic responsive *ENOD* genes (Andriankaja et al., 2007). Recent studies have further suggested that promoter sequences flanking the core GCC motif may contribute to binding selectivity of tomato ERF TFs to their target sequences (Pirrello et al., 2012). Interestingly, the crystal structure of the AP2/ERF domain of AtERF1 in the presence of its GCC box target has been determined by nuclear magnetic resonance (NMR), revealing both the folding of the DB domain and the amino

acids implicated in the interaction with the DNA (Allen et al., 1998). This study revealed that the AP2/ERF DB domain is composed of three anti-parallel β-sheets followed by an α-helix, interacting directly with the GCC box almost exclusively via the β-sheets, and through arginine (R) or tryptophan (W) residues. The key amino acids involved in the direct interaction with the DNA have been defined and are mostly conserved among ERFs (e.g., R6, R8, W10, R18, R26, and W28 in Figs. 62.2 and 62.3). Outside the ERF DB domain, protein conservation is restricted to phylogenetic groups in which conserved domains or motifs are often related to functional specialization as will be discussed subsequently.

62.3 A DIVERSE NUMBER OF ERF TFs ARE EXPRESSED IN *M. truncatula* NODULES

To gain a comprehensive view of the distribution of ERF transcription factors expressed during nodulation, ERF TFs of *M. truncatula* containing a single AP2/ERF domain were identified following the analysis of the nodule transcriptome (Table 62.2). Ninety-five out of 106 ERF TFs identified were found by RNA-seq analyses to be expressed in mature (10- and 15-day-old) nodules (Roux et al., 2014). Table 62.2 shows that the level of detected RNA-seq reads varied widely between genes, from 0.18 reads per kilobase of exon per million reads (rpkm) (ERN3) to 284.2 rpkm (for a RAP2.4 homologue). To determine the evolutionary relationship between these nodule-expressed ERFs a phylogenetic tree was constructed (Fig. 62.1). *M. truncatula* ERF TFs were classified in I–X groups, as previously defined by Nakano et al. (2006) (Fig. 62.1). The relationship among the members of a given class is not only supported by the amino acid conservation of the DB domain but also by common motifs found outside the DB domain, as well as intron positions for the very few intron-containing ERFs (in particular within groups V, VII, and X). In *M. truncatula*, ERF TFs from different classes are expressed in nodules, and this includes both DREB (groups I to IV) and ERF types (V to X) (Fig. 62.1 and Table 62.2). To date, the function of only a few of these has been studied during the nodulation process, as will be described later.

62.3.1 Group I to IV DREB-Related TFs are Mostly Associated with Abiotic Stress Responses

ERF TFs belonging to the DREB-related I–IV groups are mainly associated with plant responses to abiotic stresses (cold, drought, or salinity) and are mostly described as activators of stress-related genes containing the DRE regulatory motif (reviewed by Mizoi et al., 2012), even if certain members can also act as transcription repressors (Dong and Liu, 2010; Wind et al., 2013). Other phylogenetically related DREB TFs were also shown to have specialized functions during ABA, sugar or plastid-to-nucleus signaling (ABI4, group IV, Huijser et al., 2000; reviewed by Wind et al., 2013) or to participate in both stress and differentiation processes as is the case for the Arabidopsis RAP2.4/WIND1 (group I) TFs (Iwase et al., 2011). DREB-related transcription factors were also reported to participate in abiotic plant responses in legume plants (Li et al., 2005; Zhang et al., 2007; Gruber et al., 2009). In *M. truncatula* WXP1 and WXP2 TFs, were shown to increase drought tolerance when overexpressed in plants, and this involved the increased production of cuticular waxes, known to contribute to the regulation of water uptake and ion transport between the plant and its environment. As shown in Table 62.2, WXP1 and WXP2 are also significantly expressed in mature nodules, which raise the question of whether they may participate in the production of a similar lipophilic barrier for controlled water/ion transport in nodules.

62.3.2 Group V to X ERF-Related TFs Display Variable Functions During Plant Development, Stress or Beneficial Plant Interactions

The ERF groups (V–X) have been implicated in plant signaling pathways related to diverse biological processes, from plant development to interactions with biotic and abiotic environmental factors. The largest of these, group IX, has been often related to defense signaling during biotic plant responses (reviewed by Broekaert et al., 2006). Within this group, Arabidopsis AtERF1, AtERF2, and AtERF5 act as activators of ethylene-, jasmonate- or defense-related genes through the recognition of the GCC box. Recent studies have demonstrated that a conserved 24-amino acid "EDLL" motif is critical for the activation function of a group IX ERF TF (Tiwari et al., 2012). In contrast, group VIII members, such as Arabidopsis AtERF3, AtERF4, AtERF7, AtERF10–12, act as repressors of GCC-mediated transcription, and this involves the EAR (ERF-associated amphiphilic repression) domain conserved in a number of TF repressors (reviewed by Kagale and Rozwadowski, 2011). The sequential action of activator and repressor-type ERF TFs have been implicated in the coordination of hormonal signaling pathways to finely modulate defense response during pathogen challenge (reviewed by Broekaert et al., 2006). In *L. japonicus*, a close homolog of *AtERF1* has been implicated in induced-defense responses during nodulation (Asamizu et al., 2008). This gene, named *LjERF1*, was initially identified by a cDNA microarray analysis as being early induced in roots following inoculation with the bacterial symbiont *Mesorhizobium loti*. Subsequent over-expression studies and expression inhibition by RNA

Table 62.2 Group I to X ERF genes expressed in *Medicago truncatula* nodules.

ID	Affymetrix Probe	Best Hit *A. thaliana*	ERF Group	*Mt* gene	rpkm Nodule
Mt0005_00215	Mtr.37155.1.S1_at	AT1G64380.1	I		2.95
Mt0019_00470	Mtr.28159.1.S1_at	AT2G20880.1	I		3.03
Mt0008_00457	Mtr.40312.1.S1_at	RAP2.4-AT1G78080.1	I	*WXP1*	42.39
Mt0009_10317	Mtr.30698.1.S1_at	AT4G39780.1	I		11.92
Mt0006_10716	Mtr.43107.1.S1_at	RAP2.4-AT1G78080.1	I	*WXP2*	94.72
Mt0056_00132	Mtr.44034.1.S1_at	AT1G19210.1	II		9.64
Mt0056_00131	Mtr.29237.1.S1_at	ORA47-AT1G74930.1	II		8.3
Mt0056_00133		ORA47-AT1G74930.1	II		3.66
Mt0056_00130	Mtr.42113.1.S1_at	ORA47-AT1G74930.1	II		17.07
Mt0014_10154	Mtr.38240.1.S1_s_at	TINY2-AT5G11590.1	II		36.74
Mt0006_10776	Mtr.13273.1.S1_at	AT1G44830.1	II		13.08
Mt0008_00303	Mtr.45355.1.S1_at	AT1G44830.1	II		4.71
Mt0005_00188	Mtr.5395.1.S1_at	AT1G71520.1	II		2.56
Mt0007_10687	Mtr.51291.1.S1_at	RAP2.1-AT1G46768.1	II		13.22
Mt0002_00520	Mtr.45730.1.S1_at	RAP2.10-AT4G36900.1	II		6.17
Mt0005_00571	Mtr.40750.1.S1_at	DEAR2-AT5G67190.1	II		52.11
Mt0099_10029	Mtr.12834.1.S1_at	CBF2-AT4G25470.1	III		7.33
Mt0099_10026	Mtr.13415.1.S1_at	DREB1A-AT4G25480.1	III		7.88
Mt0099_10031	Mtr.29314.1.S1_at	DREB1A-AT4G25480.1	III		12.01
Mt0099_10028	Mtr.39012.1.S1_at	DREB1A-AT4G25480.1	III		11.15
Mt0099_10059		CBF4-AT5G51990.1	III		2.08
Mt0001_01421	Mtr.44306.1.S1_at	CBF4-AT5G51990.1	III		7.92
Mt0005_00299	Mtr.49619.1.S1_at	CBF2-AT4G25470.1	III		6.18
Mt0005_00300	Mtr.49618.1.S1_s_at	DREB1A-AT4G25480.1	III		2.86
Mt0053_00170	Mtr.38878.1.S1_at	CBF1-AT4G25490.1	III		16.42
Mt0002_00008		TINY2-AT5G11590.1	III		1.48
Mt0059_10122	Mtr.31471.1.S1_at	AT2G44940.1	III		1.84
Mt0015_00382	Mtr.32368.1.S1_at	AT3G16280.1	III		6.97
Mt0001_11451	Mtr.20988.1.S1_at	AT2G44940.1	III		1.2
Mt0001_01420		AT1G01250.1	III		1.7
Mt0053_00167	Mtr.12775.1.S1_at	AT1G12630.1	III		1.59
Mt0036_00307	Mtr.8811.1.S1_at	AT1G01250.1	III		3.71
Mt0005_00177		AT1G71450.1	III		1.46
Mt0005_10189		AT1G71450.1	III		1.33
Mt0010_10280	Mtr.12948.1.S1_at	DREB2C-AT2G40340.1	IV		9.22
Mt0026_10185	Mtr.45232.1.S1_at	DREB2A-AT5G05410.2	IV		0.69
Mt0019_00392	Mtr.9260.1.S1_at	DREB2A-AT5G05410.2	IV		4.16
Mt0121_00055	Mtr.4745.1.S1_at	DREB2A-AT5G05410.2	IV		1.27
Mt0064_00055	Mtr.1417.1.S1_x_at	AT5G25190.1	Va	*EFD*	17.42
Mt0007_10887		AT5G25190.1	Va		0.79
Mt0016_00096	Mtr.46068.1.S1_at	SHN1-AT1G15360.1	Va		2.08
Mt0095_00076	Mtr.30274.1.S1_at	ERF9-AT5G44210.1	V		3.54
Mt0009_00067		RAP2.11-AT5G19790.1	Vb	*ERN3*	0.18
Mt0033_10028	Mtr.43947.1.S1_at	RAP2.11-AT5G19790.1	Vb	*ERN2*	1.71
Mt0011_00459	Mtr.7556.1.S1_at	RAP2.11-AT5G19790.1	Vb	*ERN1*	6
Mt0007_00128	Mtr.41691.1.S1_at	CRF4-AT4G27950.1	VI		2.75
Mt0005_10243	Mtr.38693.1.S1_at	CRF4-AT4G27950.1	VI		2.22
Mt0003_01538	Mtr.49552.1.S1_at	RRTF1-AT4G34410.1	VI		2.33
Mt0003_01534	Mtr.2333.1.S1_at	RRTF1-AT4G34410.1	VI		4.23

(*continued*)

Table 62.2 (*Continued*)

ID	Affymetrix Probe	Best Hit *A. thaliana*	ERF Group	*Mt* gene	rpkm Nodule
Mt0003_01535	Mtr.20232.1.S1_at	RRTF1-AT4G34410.1	VI		19.66
Mt0003_01537	Mtr.20228.1.S1_at	RRTF1-AT4G34410.1	VI		10.17
Mt0018_10473	Mtr.12527.1.S1_at	RAP2.3-AT3G16770.1	VII		39.58
Mt0039_10183	Mtr.43313.1.S1_at	RAP2.3-AT3G16770.1	VII		284.19
Mt0028_10247	Mtr.10419.1.S1_at	RAP2.2-AT3G14230.2	VII		112.37
Mt0016_10023	Mtr.40191.1.S1_at	RAP2.2-AT3G14230.2	VII		5.48
Mt0027_00377	Mtr.37589.1.S1_at	ATERF-8-AT1G53170.1	VIII		119.84
Mt0023_00088	Mtr.26364.1.S1_at	ATERF-4-AT3G15210.1	VIII		163.44
Mt0003_11226	Mtr.26158.1.S1_at	ERF9-AT5G44210.1	VIII		68.39
Mt0023_10109	Mtr.985.1.S1_at	ERF12-AT1G28360.1	VIII		36.76
Mt0013_10760	Mtr.12544.1.S1_at	ATERF-7-AT3G20310.1	VIII		24.07
Mt0010_10370	Mtr.9081.1.S1_at	ERF3-AT1G50640.1	VIII		18.43
Mt0010_10371		ERF3-AT1G50640.1	VIII		1.25
Mt0011_00520		LEP-AT5G13910.1	VIII		1.57
Mt0004_10952	Mtr.5385.1.S1_at	ERF1-AT3G23240.1	IX		14.53
Mt0004_10951	Mtr.29300.1.S1_s_at	ATERF15-AT2G31230.1	IX		3.49
Mt0043_10018	Mtr.9223.1.S1_at	ATERF15-AT2G31230.1	IX		5.12
Mt0002_11524	Mtr.19570.1.S1_at	ERF1-AT3G23240.1	IX		2.09
Mt0004_01061	Mtr.1494.1.S1_at	ERF1-AT3G23240.1	IX		13.49
Mt0004_00866	Mtr.44496.1.S1_at	AT3G23230.1	IX		16.53
Mt0002_01809	Mtr.38122.1.S1_s_at	AT5G61590.1	IX		259.3
Mt0039_00067	Mtr.27031.1.S1_at	AT5G07580.1	IX		33.04
Mt0002_11807	Mtr.37778.1.S1_at	AT5G51190.1	IX		64.32
Mt0001_11463	Mtr.10424.1.S1_at	ATERF6-AT4G17490.1	IX		131.82
Mt0001_11464	Mtr.10425.1.S1_at	ATERF6-AT4G17490.1	IX		2.34
Mt0043_10024		AT3G23230.1	IX		3.35
Mt0023_10072		ATERF-1-AT4G17500.1	IX		1.82
Mt0001_01538	Mtr.12456.1.S1_at	ATERF-1-AT4G17500.1	IX		110.41
Mt0015_00073		ATERF13-AT2G44840.1	IX		4.88
Mt0015_10083		ATERF2-AT5G47220.1	IX		6.62
Mt0011_10607		ATERF13-AT2G44840.1	IX		12.74
Mt0011_10605	Mtr.10987.1.S1_at	ATERF2-AT5G47220.1	IX		73.76
Mt0011_10606	Mtr.10983.1.S1_at	ATERF-1-AT4G17500.1	IX		29.34
Mt0093_00008	Mtr.42129.1.S1_at	AT5G61890.1	X		14.3
Mt0027_00063	Mtr.37973.1.S1_at	AT5G61890.1	X		18.63
Mt0007_10350	Mtr.39021.1.S1_at	ABR1-AT5G64750.1	X		4.65
Mt0009_10384	Mtr.16212.1.S1_at	Rap2.6L-AT5G13330.1	X		10.66
Mt0028_00259	Mtr.44422.1.S1_at	AT3G25890.2	X		11.5
Mt0028_00263	Mtr.39336.1.S1_at	AT3G25890.2	X		6.88
Mt0007_00017	Mtr.36431.1.S1_at	Rap2.6L-AT5G13330.1	X		0.67
Mt0035_00027	Mtr.23418.1.S1_s_at	Rap2.6L-AT5G13330.1	X		0.47
Mt0002_10177	Mtr.40140.1.S1_at			*Lb1*	1249.29
Mt0017_10456	Mtr.13473.1.S1_at			*ENOD11*	58.37
Mt0005_00682	Mtr.15789.1.S1_at			*NFP*	1.17

Expression levels were determined by RNA-seq analysis of 10-day-old nodules and are represented as reads per kilobase of exon per million reads (rpkm) (mean of three biological repetitions) (Roux et al., 2014). Expression values of three symbiotic genes (*Lb1* (Ott et al. 2005), *ENOD11* (Journet et al. 2001) and *NFP* (Arrighi et al. 2006)) were included as references for highly to lowly expressed genes. Gene identifiers (first column) are from a *M. truncatula* genome sequence completed by Illumina shotgun sequencing (Roux et al., 2014).

interference experiments indicated a positive role of this TF during nodule formation. However, *LjERF1* is not expressed during nodulation-related developmental processes (rhizobium infection or nodule organogenesis) but only in bacterial inoculated roots, suggesting that *LjERF1* function is probably related to the establishment of the rhizobium symbiotic interaction, potentially by the early suppression of defense genes as suggested by the authors. Nevertheless, a direct correlation between *LjERF1* overexpression and repression of defense-related genes such as PR-10 could not be demonstrated. In *M. truncatula*, two close homologues of *LjERF1* have some level of expression in mature nodules as revealed by RNA seq analysis (Fig. 62.1 and Table 62.2) but their relative importance during nodulation has not yet been investigated.

Studies on the remaining ERF groups X, VII, VI, and V are relatively recent. Groups X, VII, and V comprise atypical ERF TFs that contain single introns in their open reading frames (Nakano et al., 2006). Group X includes ABR1 and RAP2.6 TFs which have variable functions in ABA signaling, biotic or abiotic stress responses (Pandey et al., 2005; Zhu et al., 2010; Ali et al., 2013). Group VII is a relatively small ERF group and specific members have emerged as key regulators of flooding and low oxygen responses in rice and *Arabidopsis* plants (Gibbs et al., 2011; Licausi et al., 2011; reviewed by Bailey-Serres et al., 2012). These TFs are under a tightly regulated control mechanism, in which the oxygen status determines whether the protein re-localizes to the nucleus to activate hypoxia-responsive genes or whether they are post-translationally modified for protein degradation. This refined regulation control involves the recognition of an N-terminal sequence (MCGGAI/L), that is conserved among group VII members to target protein degradation under aerobic conditions. Four *M. truncatula* ERFs expressed in nodules belong to this group (Fig. 62.1 and Table 62.2), and all contain the conserved N-terminal sequence. Within the nodule where a micro-aerobic environment is necessary for nitrogenase functioning, these ERF TFs may potentially be under a similar oxygen status-dependent post-translational control.

Group VI comprises a small subset of TFs initially reported as cytokinin responsive factors (CRF) and involved in cytokinin-regulated plant developmental processes in embryos, cotyledons, and leaves (Rashotte et al., 2006; see also Chapter 56). Recently these TFs were shown to be preferentially expressed in vascular tissues, and, as supported by mutant analyses, are also implicated in leaf vascular patterning. Nevertheless, tomato CRFs seem to be regulated not only by cytokinin but also by salt and other hormones, suggesting that CRFs may function beyond CK-regulated processes (Shi et al., 2012). At the protein level, CRFs form a unique group of ERF proteins containing a clade-specific 65 amino acid-long CRF domain that is always accompanied by an AP2/ERF DB domain. Recent comparative genomic and transcriptomic analyses have redefined this group into five clades, comprising CK-regulated and non-regulated genes (Zwack et al., 2012). Proteins from different clades comprise, in addition to the conserved N-terminal CRF and AP2/ERF domains, clade-specific motifs in the C-terminal regions. Moreover, a conserved mitogen-activated protein kinase (MAPK) phosphorylation site (SP[T/S]SVL motif) is found in many CRF proteins, and this motif is common to non-CRF proteins involved in cytokinin signaling during leaf development (Rashotte and Goertzen, 2010). Six CRF-like TFs are expressed in nodules, and two of them (Mt0007_00128 and Mt0005_10243, Table 62.2) comprise this conserved motif. Some of these factors may be potentially involved in the cytokinin signaling pathway required for nodule development in legumes (Crespi and Frugier 2008; see Chapter 56).

Finally the last ERF group V, which contains the *M. truncatula* ERF factors shown to play central roles in nodulation will be described in more detail in the following section.

62.3.3 The ERF Group V Includes the Nodule-Associated *M. truncatula* EFD and ERN TFs

M. truncatula ERF factors playing crucial roles during nodulation belong to the ERF group V. This group consists of two sub-groups Va and Vb, which are quite divergent with distinct phylogenetically conserved protein domains (Nakano et al., 2006; Figs. 62.2 and 62.3). In addition to differences in protein structure, Group Va members have a characteristic intron localized at an equivalent position in all closely related members, but that is absent in Group Vb members (Nakano et al., 2006).

62.3.4 Group Va Includes the *M. truncatula* EFD TF Required for Correct Nodule Differentiation

Group Va comprises WAX INDUCER1/SHINE1 (WIN1/SHN1) and related clade members that when overexpressed in *Arabidopsis* lead to enhanced accumulation of epidermal waxes (Aharoni et al., 2004; Broun et al., 2004). Interestingly the same phenotype is observed in a gain-of function *SHN1* mutant that presents brilliant shiny leaves due to excessive wax production. WIN1/SHN1 and closely related clade members are thought to display similar functions, and this is supported by the high conservation of the DB and additional C-terminal CMV-1 and CMV-2 domains (Nakano et al., 2006, Fig. 62.2). Nevertheless, respective genes show not only overlapping but also specific expression profiles suggesting a certain degree of protein specialization (Aharoni et al., 2004). Recently, a synthetic microRNA approach used to simultaneously silence the three *SHINE* (*SHN*) clade

members of *Arabidopsis* revealed that these TFs act redundantly to regulate the surface of flower organs by controlling not only cuticular lipid metabolism but also the epidermal cell wall (Shi et al., 2011). In rice the *WIN1/SHN1* homolog *OsWR1* also acts as a positive regulator of wax synthesis and has been involved in drought tolerance (Wang et al., 2012). In barley, a *WIN1/SHN1* homolog has been identified as the gene responsible for conferring organ adhesion in covered barley, potentially by controlling lipid production (Taketa et al., 2008).

M. truncatula possesses a *WIN1/SHN1* homolog that shares highly conserved protein domains with the *Arabidopsis* orthologs, suggesting potentially related protein functions (Figs. 62.1 and 62.2). This similarity includes the N-terminal region comprising the DB domain and the CMV-1 /CMV-2 domains. *M. truncatula* also contains another group Va member referred to as EFD (ethylene response factor required for nodule differentiation) that has been associated with the development of nitrogen fixing nodules (Vernié et al., 2008). Despite relatively high conservation in both the N-terminal region and DB domain (~84%), divergences are observed in the remaining C-terminal domains. While the CMV-1 and CMV-2 domains are conserved among WIN1/SHN1 orthologs of *M. truncatula* and *Arabidopsis*, they are only partially or poorly conserved in EFD and EFD-like TFs from different legumes (Fig. 62.2). In contrast, these EFD-like TFs share highly conserved domains that are likely to be related to specific functions of EFD.

EFD was identified following transcriptomics and subtractive hybridization strategies as being up-regulated in rhizobial-induced root nodules (El Yahyaoui et al, 2004; Godiard et al, 2007). To further understand the function of *EFD* during nodulation, a deletion mutant that completely abolishes *EFD* expression was obtained following reverse screening of a fast-neutron *M. truncatula* mutant population (Rogers et al., 2009, Vernié et al., 2008). This mutant showed an abnormally high number of nodules, often accompanied by the presence of multiple infection sites developing later into multilobed nodule structures. These experiments coupled with RNA interference and overexpression approaches support a role of EFD in the regulation of nodule number. In this context, EFD has recently being implicated in the signaling pathway involved in autoregulation of nodulation (AON) in *M. truncatula*, since *EFD* expression is up-regulated in roots over-expressing a key AON CLE peptide regulator named *MtCLE12* (Saur et al., 2011).

In addition to its role in the regulation of nodule density, *EFD* is also implicated in the control of nodule differentiation since *efd* nodules present an abnormally enlarged infection nodule zone II containing numerous and defective infection threads, which is often associated with a severe alteration in their capacity to differentiate into functional nitrogen-fixing nodules. These data suggest an important role for *EFD* in symbiosome formation which requires a symbiotic signaling pathway controlled by IPD3 (Ovchinnikova et al., 2011). Consistent with the roles assigned to this factor, the expression of *EFD* is mainly observed in young nodules and in zone II of mature nodules. Finally, a transcriptomic strategy focused on mutant and over-expressing *EFD* roots led to the identification of a cytokinin A-type response regulator-encoding gene named *MtRR4* as a potential direct target of *EFD*. Further transactivation assays came to support this hypothesis, since the nuclear-localized EFD protein was able to activate the transcription of *MtRR4* when transiently expressed in *Nicotiana benthamiana* leaves. While *MtRR4*, involved in cytokinin signaling, has been related to EFD function during nodule differentiation, *MtN19* has been suggested as a potential target of EFD in the negative feedback inhibition of nodule initiation (Moreau et al., 2011).

62.3.5 Group Vb Members Display Specialized Functions in Root Tissues and Includes the *M. truncatula* ERN TFs Involved in Symbiotic Signaling and Root Infection

Group Vb is represented by one unique TF in Arabidopsis and three phylogenetically related members in *M. truncatula*. While the legume TFs are involved in root nodulation, the *Arabidopsis* member RAP2.11 has been recently implicated in root responses to low levels of phosphate (Kim et al., 2012). Indeed, *RAP2.11*, identified following an activation-tagging strategy, was shown to directly mediate the transcriptional activation of a low phosphate responsive *AtHAK5* reporter gene in *A. thaliana* roots. This activity requires a modified GCC motif (GCCGGC) found within the promoters of *AtHAK5* and other genes up-regulated in *RAP2.11*-overexpressing plants, which includes genes required for the production of reactive oxygen species (ROS), ethylene and calcium signaling.

The nodulation-associated ERN1 (ERF required for nodulation) TF was first discovered through a genetic screen, as a gene essential for nodule formation in *M. truncatula* (Middleton et al., 2007). The deletion knockout mutant of ERN1 named *bit1-1* (for branched infection threads) was strongly affected in its ability to form nodules and to initiate rhizobial infection which was primarily blocked at the root surface despite certain abnormal infections initiated in root hairs. A similar nodulation-defective phenotype was observed in other *M. truncatula* or *P. sativum ern1* mutant alleles, carrying either Tnt1-insertion or EMS point mutations (Tsyganov et al., 2002; Middleton et al., 2007; Pislariu et al., 2012). These results, together with complementation studies, confirm the importance of ERN1 for nodule formation (Middleton et al., 2007; Cerri et al., 2012; see Chapter 59). Further analyses revealed that the *ern1* knockout mutant was also affected during early

symbiotic stages, because rhizobial-secreted symbiotic NF signals were no longer able to activate the expression of the NF-responsive *ENOD11* marker gene (Middleton et al., 2007). In this NF-responsive signaling pathway, ERN1 is positioned downstream of GRAS-type TFs NSP1/NSP2 (nodulation signaling pathway), which together are able to directly regulate *ERN1* (Hirsch et al., 2009; Cerri et al., 2012).

The role of ERN1 during early NF signaling was clarified by the discovery, following a yeast one-hybrid screen, that ERN1 was able to directly bind to the *ENOD11* promoter through a ~30 bp NF-box-responsive promoter sequence, necessary and sufficient to drive NF-elicited gene expression in *M. truncatula* roots (Boisson-Dernier et al., 2005; Andriankaja et al., 2007). In addition to ERN1, the phylogenetically related ERN2 and ERN3 TFs also bind to the *ENOD11* NF-box and this requires a conserved GCC-rich motif (GCAGGC) that strikingly resembles the GCC (GCCGGC) motif recognized by the *A. thaliana* RAP2.11 ortholog (Andriankaja et al., 2007; Kim et al., 2012, L. Frances and F. de Carvalho-Niebel, unpublished data). These TFs have very similar DB domains (~90% sequence identify) and this conservation is also observed among ERN-like TFs from other leguminous (*L. japonicus*, *P. sativum*, *Phaseolus vulgaris*, *Glycine max*) or non-leguminous plant species (*Populus alba*, *Oryza sativa*) (Fig. 62.3; Middleton et al., 2007). Outside the DB domain, the *A. thaliana* RAP2.11 and the *M. truncatula* ERN1 and ERN2 TFs share the phylogenetically conserved CMV-3 and CMV-4 domains defined by Nakano et al. (2006). However, sequence conservation within these domains is particularly high among legume members suggesting that they have more closely related functions. In line with this, most legume sequences, with the exception of ERN3, comprise a rosid-specific region common to poplar but not conserved in *Arabidopsis* (Fig. 62.3; Middleton et al., 2007). In *M. truncatula,* ERN1 and 2 are functionally related and both act as transcription activators. On the other hand, the more divergent ERN3 TF is unable to activate transcription and potentially act as a transcriptional repressor (Fig. 62.3; Andriankaja et al., 2007). Recent studies have demonstrated that ERN1, presumed to be derived from ERN2, can be functionally replaced by ERN2, reinforcing the likelihood of similar biological functions for these related factors (Young et al., 2011; Cerri et al., 2012).

Genetic and expression studies suggest that ERN1 and ERN2 play partially redundant functions during early stages of signaling and rhizobial infection. Indeed, the leaky phenotype of the *ern1* deletion mutant in terms of gene expression during NF signaling and rhizobial infection could be due to the presence of ERN2 (Mitra et al., 2004; Middleton et al., 2007; Cerri et al., 2012). Functional redundancy among ERN1 and 2 is reinforced by their overlapping expression profiles during certain stages of NF signaling and rhizobial infection. However, specific differences in their spatio-temporal expression profiles and in the dynamics of protein fusion accumulation *in vivo* also suggest specialized functions for these factors. ERN1 appears to be involved in stages preceding and accompanying infection while ERN2 would be involved in certain stages of infection (Cerri et al., 2012). During these early symbiotic stages, ERN1 mRNA also seems to be under translational control by selective recruitment to polysomes following bacterial inoculation (Reynoso et al., 2012).

In contrast to ERN2, ERN1 is expressed in nodule primordial cells in the root cortex that will further develop into nodule structures. This is in line with the *ern1* mutant phenotype that is deficient for nodule formation, and the fact that a constitutively active form of DMI3 kinase responsible for the formation of spontaneous rhizobia-free nodules, fails to form any nodules in the *ern1* mutant background (Middleton et al., 2007). Nevertheless, ERN1 does not seem to be absolutely required for initial root cortical cell divisions, but acts at a subsequent preinfection stage during nodule formation (Middleton et al., 2007; Cerri et al., 2012; see Chapter 59). Cytokinins, well known for their positive role in nodule organogenesis may be directly involved in the regulation of *ERN1* during this symbiotic stage via the potential regulation of *ERN1* by B-type cytokinin response regulators (Plet et al., 2011; Ariel et al., 2012). Finally, recent transcriptomic studies have highlighted the fact that ERN TFs may also play important roles during root endosymbiotic interactions with arbuscular mycorrhizal (AM) fungi, and in particular ERN2, which is up-regulated in AM inoculated roots (Young et al., 2011; Hogekamp et al., 2011). Future analyses of single or double ERN1/2 mutants will help to elucidate the relative importance of these two related TFs in root endosymbioses.

62.4 CONCLUDING REMARKS

The AP2/ERF superfamily constitutes one of the largest TF families, widely represented in plant species. Members of the ERF group, containing a unique AP2/ERF DB domain, are the major group within the superfamily, displaying roles in various biological processes such as the root nodule symbiosis. So far, only a limited number of ERF TFs have been shown to play essential roles during nodule formation and to date this is restricted to members of ERF group V. Despite belonging to the same phylogenetic group, ERN-type and EFD TFs display important structural differences and have quite different functions during the nodulation process. While ERN1 and 2 paralogs play critical roles during early symbiotic signaling and rhizobial infection, EFD functions at a later stage controlling nodule number and nodule differentiation (see Chapter 59). Recent RNA seq analyses have revealed that a large number of ERF TFs are expressed in mature nitrogen-fixing nodules, suggesting that ERFs from

different functional categories may be associated with nodule development. This myriad of potential candidates opens new perspectives for understanding the full contribution of AP2/ERF-mediated transcriptional reprogramming to the establishment of the root nodule symbiosis.

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Chapter 63

Identification of *Medicago truncatula* Genes Required for Rhizobial Invasion and Bacteroid Differentiation

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63.1 INTRODUCTION

Legumes have the ability to develop a root endosymbiosis with soil bacteria, termed rhizobia and arbuscular mycorrhizal (AM) fungi. These capacities confer selective advantages for legumes to grow in soils of low nutrient content and render them the primary source of plant proteins for livestock feeding and human nutrition and indispensable crops in sustainable agriculture. In both nitrogen-fixing and AM associations, the microbial partners modulate the growth and development of legumes to be able to colonize the plant tissues and establish a symbiotic interaction.

The legume–rhizobial symbiotic interaction accounts for the major portion of the biologically fixed nitrogen in the ecosystem. Symbiotic nitrogen fixation takes place in root – or sometimes stem – nodules, and nodule development is initiated by the host-compatible symbiotic bacterial partner. The interaction commences following a mutual recognition process of the host plant and rhizobial signal molecules. The key signal molecule is the nodulation (Nod) factor (NF) which governs the parallel developmental processes of bacterial infection initiated in the root epidermis and nodule organogenesis activated in the root cortex (Oldroyd and Downie, 2008; see Chapters 51, 59). The attachment of rhizobia to root hairs induces the formation of a tubelike structures called infection threads (IT) which extend through the root epidermis toward the nodule primordia wherein they ramify and bacteria are released from the ITs into the cytoplasm of nodule cells (Jones et al., 2007). Bacteria are surrounded by a plant-derived membrane within the plant cells forming new subcellular compartments called symbiosomes (see also Chapters 31, 67). Bacteria in the symbiosomes divide and differentiate into their symbiotic forms (Mergaert et al., 2006), called bacteroids resulting in colonized and fully packed nodule cells with bacteroids. Similarly to rhizobia, infected nodule cells also undergo cell enlargement-coupled differentiation (Vinardell et al., 2003) to form the mature nodule. As a result of a compatible interaction between the symbiotic partners, a functional root nodule is developed wherein the plant partner supplies the bacteroids with photosynthetic products in exchange for ammonia converted from atmospheric nitrogen. *Medicago truncatula* forms cylindrical shaped indeterminate nodules characteristic for temperate legumes (Vasse et al., 1990). The mature *M. truncatula* nodules display a developmental gradient of cells, creating the zones of the typical indeterminate nodules. A group of cells forms the persistent meristematic region (zone I) at the nodule apex wherein the continuous

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cell division produces new nodule cells. Bacteria are released from the IT into the cells of the infection zone (zone II), and the differentiation of rhizobia and plant cells is started in this region. The differentiation programs are completed in the few cell layer of the interzone (zone II–III). The major part of the mature nodule is composed of the symbiotic zone (zone III) wherein nitrogen fixation takes place. The basal part of older nodules contains a senescence region (zone IV) wherein bacteroids and nodule cells undergo degradation.

The development of the model legume systems *M. truncatula* and *Lotus japonicus* and their substantially completed genome sequences (Sato et al., 2008; Young et al., 2011) greatly advances the molecular studies of legume–microbe symbiotic interactions. Mutants impaired in certain biological processes provide a valuable tool to dissect gene function. Several mutant populations have been produced to identify symbiotic genes in model legumes applying chemical treatment (Benaben et al., 1995; Szczyglowski et al., 1998; Penmetsa and Cook, 2000; Perry et al., 2003; Le Signor et al., 2009), ionizing irradiation (Starker et al., 2006; Rogers et al., 2009) and exploiting the mobilization activity of transposons (Schauser et al., 1999; Tadege et al., 2008; Rakocevic et al., 2009; Pislariu et al., 2012; see Chapter 83). The genetic dissection of the establishment of rhizobial and AM symbiosis in both model legumes has identified many of the key components of the symbiotic signaling (Sym) pathway (also known as CSP or CSSP; recently reviewed by Kouchi et al., 2010; Madsen et al., 2010; Murray, 2011; Venkateshwaran et al., 2013; Oldroyd, 2013; see Chapters 42, 55, 59, 110). In addition, plant genes functioning in the regulation of nodule number (Nishimura et al., 2002; Krusell et al., 2002; Schnabel et al., 2005; Penmetsa et al., 2008), infection (Arrighi et al., 2008; Kiss et al., 2009; Yano et al., 2009), bacterial differentiation (Wang et al., 2010), maintenance (Kumagai et al., 2007; Bourcy et al., 2013), and transport (Krusell et al., 2005; Bagchi et al., 2012) have been also identified by analyzing mutants defective for nodulation (see also Chapter 59).

Fast neutron (FN) bombardment can provide an efficient method to generate knockout mutants in plants (Li et al., 2001; 2002; Rogers et al., 2009; Bolon et al., 2011). Compared to chemical mutagenesis which induces point mutations and to transposon or T-DNA insertion mutagenesis, FN irradiation generally induces deletions. As detected in *M. truncatula* mutant loci, the deletions can range from few base pairs (Mitra et al. 2004) to tens of kilobase pairs (Ane et al., 2004; Middleton et al., 2007). In some cases, the deletion event is coupled with point mutations, as it was found in the *ipd3-1* allele (Horvath et al., 2011), or the FN irradiation generated other rearrangements (*lin-3* allele; Kiss et al., 2009).

In this study, we report the result of a genetic screen to identify symbiotic mutants of *M. truncatula* blocked in nodule invasion, persistence of rhizobia, and nodule function. FN-bombarded and ethyl methanesulfonate (EMS)-mutagenized *M. truncatula* seed populations were screened for nodulation defects, and nonnitrogen-fixing (Fix-) mutants were selected from the candidate mutants and characterized further (Domonkos et al., 2013). In this chapter, we describe the genetic analysis of the *M. truncatula* symbiotic mutants isolated and the phenotypic characterization of the mutants defective in nodule function. Allele tests were carried out to disclose new alleles of known symbiotic genes and identify novel complementation groups. Microscopical and gene expression analyses of Fix- mutants were performed to reveal which stages of the symbiotic interaction were blocked and to analyze the potential function of the affected genes.

63.2 MATERIALS AND METHODS

63.2.1 Plant Material, Growth Conditions, and Bacterial Strains

The large-scale symbiotic screen was carried out on FN-bombarded and EMS-mutagenized seed populations of *M. truncatula* Jemalong line (FN) and genotype A17 of *M. truncatula* (EMS) as described in Domonkos et al. (2013). The seeds of the Jemalong genotype of *M. truncatula* were exposed to FN radiation as described by Rogers et al. (2009). For the symbiotic phenotype screen, nodulation tests, and expression studies, the *M. truncatula* seeds were scarified, sterilized, washed, and imbibed as described in the *M. truncatula* handbook (http://www.noble.org/MedicagoHandbook). Sterilized seeds were vernalized for 5–7 days at 4 °C and thereafter germinated on inverted agar (0.8% water agar) plates in the dark at room temperature. For the symbiotic screen, mutagenized and control wild-type seedlings were grown in 1:1 mixture of Terragreen and sand as described by Marsh et al. (2007). Four-day-old *M. truncatula* seedlings were inoculated with the robust *Sinorhizobium meliloti* strain B1 in the primary screen, and plants were evaluated for their symbiotic phenotype 5–6 weeks postinoculation. Confirmation screening experiments were carried out in the same growing substrate in walk-in growth chambers as described by Marsh et al. (2007).

For phenotypic characterization, ineffective nodulation mutants and wild-type plants were grown on either square plates containing buffered nodulation media (BNM) supplemented with 0.1 μM L-α-(2-aminoethoxyvinyl)-Gly (AVG) (Sigma-Aldrich, St. Louis) or in trays containing a 3:1 mixture of zeolite substrate (Geoproduct Kft., Mád, Hungary) and sand. Four-day-old *M. truncatula* seedlings were inoculated with *S. meliloti* strain 1021 carrying the *hemA*::*lacZ* reporter construct (pXLGD4; Boivin et al., 1990) or *S. meliloti* strain CSB357 containing the P*nifH*::*uidA* fusion (Starker et al., 2006). *S. meliloti* cultures were grown in liquid TA, and bacteria were pelleted at log phase and

resuspended in liquid BNM. A final dilution 1:50 (OD_{600} 0.03–0.1) of bacterial suspension was used for inoculation by flooding the roots on plate or adding 500 µl suspension to each plant in the tray. Plants on plates and trays were grown in growth chambers using the same light and temperature conditions as described earlier by Horvath et al. (2011).

Genetic crossings between symbiotic mutants and genotypes were carried out according to the method described by Chabaud et al. (2007). *ipd3-1/dnf5-1* double mutant plants were selected from the F2 population of the cross of *ipd3-1* and *dnf5-2* plants with PCR using gene-specific oligonucleotides able to detect the deletions in *IPD3* (Horvath et al., 2011) and *DNF5* (unpublished result) genes.

63.2.2 Microscopic Analysis

For microscopic analysis, nodules were harvested 3 weeks postinoculation with *S. meliloti* 1021 (pXLGD4) or *S. meliloti* strain CSB357 and fixed with 4% formaldehyde in 1xPBS (pH 7.4) for 30 min on ice and rinsed 3 × 15 min in 1xPBS (pH 7.4). The nodules were embedded in 5% agarose (SeaKem® LE Agarose, Lonza), and 70 µm thick longitudinally sections were prepared using a MICROM HM 650V Vibrotom. To visualize β-galactosidase activity, sections were incubated in a staining solution containing 50–50 mM potassium ferricyanide and potassium ferrocyanide and 0.08% X-gal (Fermentas, Lithuania) in 1xPBS (pH 7.4) for 30 min at room temperature. Stained nodule sections were observed under an Olympus BX41M microscope with 10x and 20x objectives, and images were captured using an Olympus Camedia E-10 digital camera. GUS staining was carried as described by Horvath et al. (2011).

To analyze the bacteroid morphology, nodule sections were stained in 1xPBS (pH 7.4) containing 5 µM SYTO13 (Invitrogen, Eugene, Oregon) for 20 min and rinsed with 1xPBS.

Confocal laser scanning microscopy was performed using an Olympus FluoView FV1000 confocal laser scanning microscope (Olympus Life Science Europa GmbH, Hamburg, Germany). Microscope configuration was as follows: objective lenses, UPLSAPO 10x (dry, NA:0.4), UPLFLN 40x (oil, NA:1.3), and UPLSAPO 60x (oil, NA:1.35); sampling speed, 4 µs/pixel; line averaging, 2x; scanning mode, sequential unidirectional; excitation, 488 nm (SYTO13); laser transmissivity, 5%; main dichroic beam splitter, DM405/488; intermediate dichroic beam splitter, SDM 490; and emission filter, 505–530 nm.

63.2.3 Gene Expression Analyses

For quantitative RT-PCR experiments, *M. truncatula* plants were grown on plates using the same conditions as described for the microscopic analysis. *Medicago* roots or nodulated roots were harvested 14 days postinoculation, and the total RNA was extracted using the TRI Reagent (Sigma, United States) following the manufacturer's protocol. The RNA samples were treated with RQ1 DNase I (Promega, United States) according to the manufacturer's instructions. RNA was cleaned up by using the RNeasy Mini Kit (QIAGEN, DE). Genomic DNA-free total RNA was quantified using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, United States) and checked for quality by gel electrophoresis.

Complementary DNA was prepared from 1 µg total RNA with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, United States) using oligo-dT primers according to the manufacturer's instructions. Quantitative RT-PCR was performed using the MiniOpticon™ System (Bio-Rad, United States) employing Bio-Rad CFX Manager software 3.0. Maxima™ SYBR Green Master Mix (Thermo, United States) was used to monitor double-stranded (ds) DNA synthesis in 48-well plates. The final primer concentration of each gene-specific primer was 100 nM. The PCR conditions were as follows: a single cycle of 94°C for 10 min was followed by 45–50 cycles of 94°C for 15 s, 58–60°C for 20 s, and 72°C for 20 s. Following each PCR amplification, a melting curve was run to check genomic DNA contamination. To generate a melting profile, fluorescence of the samples was measured repeatedly as the temperature was gradually increased from 60 to 95°C over 20 min after complete denaturation. Primer–dimer formation was estimated by running a control without template DNA. Results were expressed as a threshold cycle (C_T) value, which was averaged from three replicate reactions. For normalization, the C_T value of the reference gene was subtracted from the C_T value of the gene of interest (ΔC_T). $\Delta\Delta C_T$ values were calculated by subtracting the ΔC_T of the wild-type sample from the ΔC_T value of the different mutants. Fold induction ($2^{\Delta\Delta C_T}$) of three independent experiments were averaged and plotted using SE. Primer sequences used for qPCR are published in the study of Domonkos et al. (2013). A gene with an ubiquitin domain was used as a reference gene, and its intron sequence was utilized for checking genomic DNA contamination of cDNA samples, as suggested by Kakar et al. (2008).

63.3 RESULTS

63.3.1 Developing a Symbiotic Nitrogen Fixation Mutant Screen and Identification of *M. truncatula* Mutants Defective in Nodule Function

In order to identify mutants defective in rhizobial invasion and bacteroid differentiation, we aimed to screen *M. truncatula* plants able to develop ineffective root nodules

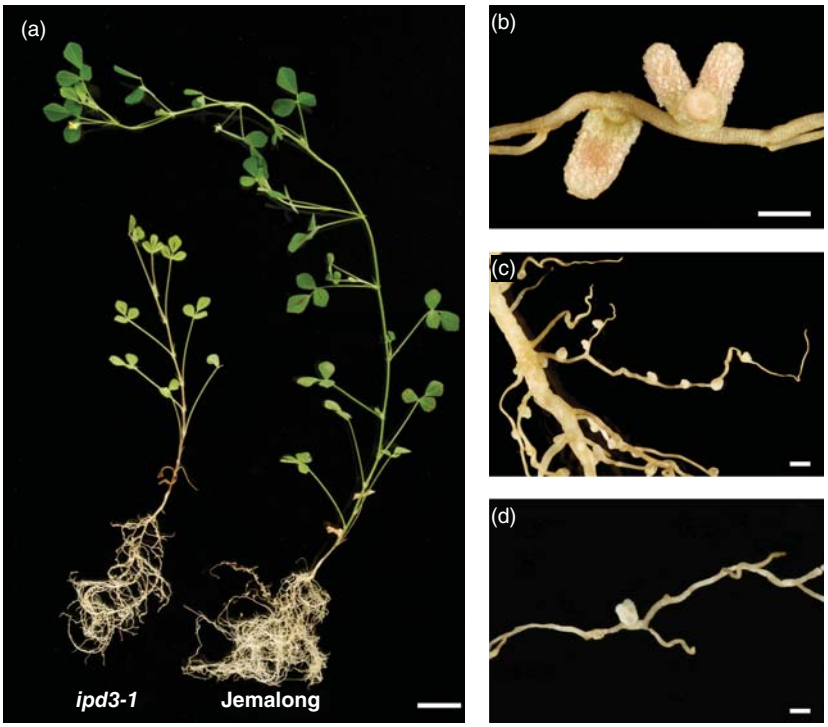

Figure 63.1 Nodulation phenotype of ineffective nodulation (*ipd3-1*) and wild-type *M. truncatula* plants 4 weeks postinoculation (wpi) with *S. meliloti* 1021. Plants were grown in zeolite/sand substrate. Ineffective mutants display retarded growth and showed the characteristic symptoms of nitrogen deficiency. Wild-type plants developed pink cylindrical nodules (b). Both spherical (c) and elongated (d) white nodules were found on ineffective *ipd3-1* mutant roots. Scale bars: 2 cm in (a), 1 mm in (b)–(d).

(nodules without the ability to fix nitrogen). A large-scale forward genetic screen was developed using FN-bombarded and EMS-mutagenized *M. truncatula* Jemalong populations (Domonkos et al., 2013). Approximately 38,000 seedlings of about 600 M2 families (Marsh et al., 2007) were grown in media of low N content and screened for symbiotic phenotype 5–6 weeks postinoculation with wild-type *S. meliloti* strain B1 (Oldroyd and Long, 2003). Plants defective in symbiotic nitrogen fixation were identified with visual screening for nitrogen deficiency symptoms (yellowish leaves, purplish stem due to anthocyanin accumulation, stunted growth) and the ability to develop nodules (Fig. 63.1). The progenies of the putative mutants were subjected to a second round of tests to confirm their symbiotic phenotype, and finally, seven ineffective nodulation (Fix-) mutants developing small white nodules on roots indicating the absence of leghemoglobin were selected for further analysis. These mutants were initially designated as 5L, 7Y, 9F, 11S, 12AA, 13U, and 14S.

63.3.2 Genetic Analysis of Ineffective Symbiotic Mutants of *M. truncatula*

The growth habit of the ineffective mutants was tested in medium supplemented with combined nitrogen to prove that the nitrogen deficiency was the result of impaired nitrogen fixation. The growth habit of the seven ineffective plants was similar to wild-type plants under nonsymbiotic conditions indicating their defects in symbiotic nitrogen fixation rather than in nitrogen uptake or metabolism. The seven Fix- mutants were backcrossed to the wild-type Jemalong and also crossed to the A20 genotype to analyze the inheritance of the Fix- phenotypes and to develop F2 mapping populations. The segregation ratios of the Fix- phenotype (Table 63.1) in the F2 progeny of these crosses indicated that all the new ineffective mutants segregated as single recessive loci. Crosses between the mutant plants and to the previously identified ineffective *dnf* mutant series of *M. truncatula* (Starker et al., 2006) were carried out to define how many independent loci were represented by the seven ineffective-nodulating mutants. The allelism test revealed two novel complementation groups. For the reasons described in the following, we called the responsible locus of one of them, *IPD3*, with the mutant allele referred to as *ipd3-1* (Horvath et al., 2011) and the other as *dnf8* (Table 63.2). The ineffective symbiotic phenotype of the F1 hybrids of 14S and *dnf5* revealed an allelic relationship, and therefore, 14S is hereafter termed *dnf5-2*.

Four additional Fix- mutants were partially analyzed. The allelism tests revealed that 5L and 11S carried mutations at the same locus, but incomplete analyses of these along with 7Y and 13U meant that we were not able to define with allelism tests alone whether these represent new genetic loci or alleles of already defined *DNF* loci. However, the allelism tests performed to date reveal no similarities with the *DNF* loci tested (Table 63.2). In order to define whether mutants 5L/11S, 7Y, and 13U represented new mutants or alleles of

Table 63.1 Segregation analysis for the ineffective symbiotic phenotype of the seven selected ineffective mutants isolated in the symbiotic mutant screen

Crosses	Number of Plants		Segregation Ratio of Wild Type and Mutant Alleles	χ^2
	Fix+	Fix−		
5L × A17	42	11	3.8:1	0.594
5L × A20	85	31	2.7:1	0.183
11S × A17	50	12	4.2:1	1.053
11S × A20	136	30	4.5:1	**4.249***
7Y × A17	46	34	1.4:1	**13.06*****
7Y × A20	132	47	2.8:1	0.151
ipd3-1 × A17	40	13	3.1:1	0.006
ipd3-1 × A20	318	132	2.4:1	**4.051***
12AA × A17	43	5	8.6:1	**5.44****
12AA × A20	186	86	2.2:1	**6.35****
13U × A17				
13U × A20	293	100	2.9:1	0.041
14S × A17	34	19	1.8:1	3.327
14S × A20	555	156	3.5:1	3.548

Segregation data presented for backcross and F2 segregation populations. χ^2 values were calculated based on the 3:1 segregation ratio; $P > 0.05$; * at 0.025 significant level; ** at 0.01 significant level; *** at 0.005 significant level.

Table 63.2 The results of the allelism tests between the ineffective mutants identified in this study and known symbiotic mutants of *M. truncatula*

Recipient / pollinator	*dnf1*	*dnf2*	*dnf3*	*dnf4*	*dnf5*	*dnf6*	*dnf7*	*ipd3-1*	*dnf5-2*	*dnf8* (12AA)	7Y	13U	5L	11S
dnf1											+ (4/2)		+ (7/3)	
dnf2												+ (16/4)		+ (6/2)
dnf3														
dnf4											+ (8/3)		+ (3/1)	+ (10/3)
dnf5									– (5/2)				+ (4/1)	
dnf6														
dnf7										+ (4/2)	+ (4/1)			
ipd3-1	+ (8/2)	+ (8/3)	+ (9/3)	+ (10/3)	+ (7/2)	+ (8/2)	+ (7/2)			+ (3/1)		+ (5/2)	+ (5/1)	+ (7/2)
dnf5-2	+ (6/4)	+ (5/4)	+ (6/1)	+ (11/3)	– (20/4)	+ (14/3)	+ (18/3)	+ (16/5)						
dnf8 (12AA)	+ (2/1)	+ (10/3)	+ (23/5)	+ (4/2)	+(6/2)	+ (9/3)	+ (12/3)	+ (11/3)	+ (7/3)		+ (4/2)			
7Y							+ (15/4)	+ (4/2)		+ (2/1)		+ (8/2)		
13U		+ (3/2)							+ (4/2)	+ (4/1)				+ (5/2)
5L		+ (11/3)					+ (4/1)				+ (7/2)			– (6/2)
11S	+ (7/2)								+ (9/3)	+ (4/2)	+ (6/2)	+ (6/2)	– (16/3)	

The plus sign indicates that progeny displayed wild-type symbiotic phenotype. The minus sign indicates that F1 hybrid plants displayed nitrogen deficiency symptoms under symbiotic conditions. Numbers in parenthesis represent the plants scored for symbiotic phenotype and the numbers of pods from which seeds originated. The allelic relationships are highlighted with gray.

known complementation groups, further allelism tests or genetic mapping of the Fix- loci are required.

63.3.3 Macroscopic Characterization Revealed That Ineffective Mutants Are Arrested in Different Stages of Symbiotic Nitrogen Fixation

In order to analyze the histology of the mutant nodules, to determine whether rhizobia were released in the mutant nodule cells and assess at which stages the nodule development was arrested in the Fix- mutants, *M. truncatula* wild-type and mutant plants were inoculated with *S. meliloti* 1021 (pXLGD4) which constitutively expresses the *LacZ* reporter gene [*hemA*::*lacZ*; Boivin et al., 1990] and with *S. meliloti* strain CSB357 containing a P*nifH*::*uidA* fusion (Starker et al., 2006).

The isolated ineffective symbiotic mutants developed exclusively white spherical or cylindrical nodules except *dnf8* on which a few pale pinkish nodules could be observed occasionally. To visualize the presence of bacteria in the nodules, we stained thin longitudinal sections of 21-day-old nodules following inoculation for β-galactosidase activity. The extent of bacterial colonization in the nodule zones was examined by light microscopy, and micrographs of sections are shown in Figure 63.2a–h. The X-gal staining showed the typical zonation (Vasse et al., 1990) of a fully developed indeterminate nitrogen-fixing nodule on wild-type *M. truncatula* Jemalong plants (Fig. 63.2a). The majority of the nodules formed on *ipd3-1* roots were spherical with abnormal nodule apices (Fig. 63.2b), but a small number of nodules developed into elongated cylindrical structures (Fig. 63.3d, e) (Horvath et al., 2011). Neither class of *ipd3-1* nodules contained cells with released bacteria, indicating an essential function of *IPD3* for bacterial release. In contrast to *ipd3-1*, the *dnf5-2* nodules contained cells harboring bacteria (Fig. 63.2c), but no characteristic zonation of the indeterminate nodules was observed in *dnf5-2* nodules.

The other Fix- mutants developed more or less elongated nodules (Fig. 63.2d–h), but the nodule zonation or occupancy by rhizobia was impaired in these mutants. The nodules of mutant 7Y showed extremely low bacterial occupancy; bacteria could be detected only in a few cells of the infection zone, and only a few IT were present in the nitrogen fixation zone (Fig. 63.2d). Moreover, an extensive brown pigmentation was observed throughout the nodule. Sporadic brown pigmentation was also present in 5L and 11S nodules (Fig. 63.2e, f) and to a lesser degree in 13U nodules (Fig. 63.2h). We believe that this brown pigmentation may be associated with the senescence of cells within the nodules of these Fix- mutants. Mutants 5L and 11S developed nodules with a narrow infection zone containing several infected cells, but the infected cells in the intermediate and nitrogen fixation zone appeared to be degraded (Fig. 63.2e, f). In *dnf8* nodules, infected cells occurred in the interzone, but no infected cells and only a few IT were found in the nitrogen fixation zone (Fig. 63.2g). Similarly, the 13U nodules showed bacteria within the cells of the infection zone but low levels of infection within the nitrogen fixation zone (Fig. 63.2h).

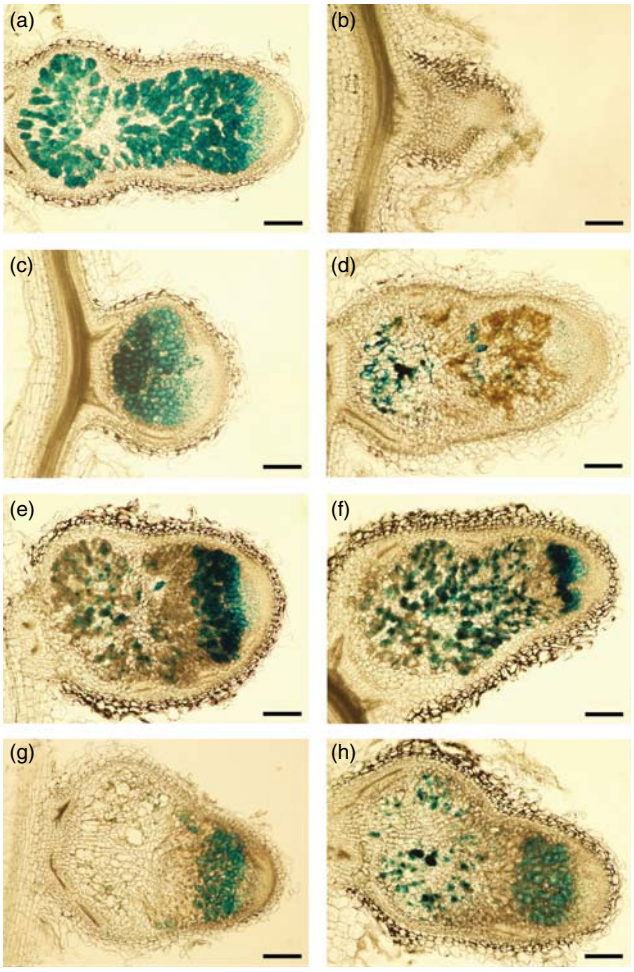

Figure 63.2 Rhizobial infection of nodules developed on roots of wild-type (a) and ineffective nodulation mutants (b–h). Nodules were harvested 3 weeks postinoculation with *S. meliloti* 1021 strain expressing the *lacZ* gene. 70 µm thick nodule sections prepared with a vibration microtome were stained with X-gal to detect β-galactosidase activity. (b–h) Nodules of *ipd3-1* (b), *dnf5-2* (c), 7Y (d), 5L (e), 11S (f), *dnf8* (g), and 13U (h) mutants show various bacterial colonization phenotypes. Bars represent 200 µm.

Analysis of double mutant phenotypes constitutes a powerful tool to analyze the functional relationship between genes involved in the same biological process. Although the nodulation phenotype of *ipd3* and *dnf5* single mutants suggested that the function of IPD3 precedes DNF5, we analyzed the nodule phenotype of *ipd3/dnf5* double mutants following inoculation with *S. meliloti* 1021 (pXLGD4).

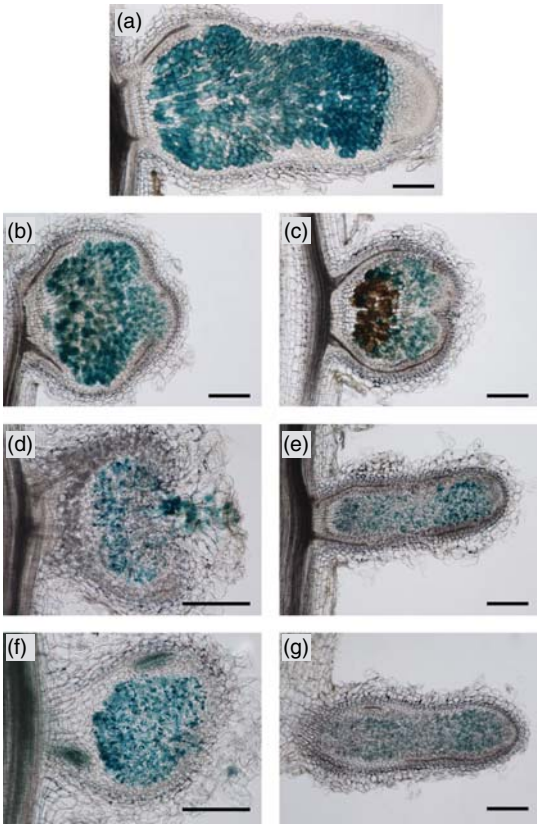

Figure 63.3 Rhizobial infection of nodules developed on roots of wild-type (a), single *dnf5-2* (b and c), and *ipd3-1* (d and e) and double *ipd3-1/dnf5-2* mutants (f and g). Nodules were harvested 3 weeks postinoculation with *S. meliloti* 1021 strain expressing the *lacZ* gene. 70 µm thick nodule sections prepared with a vibration microtome were stained with X-gal to detect β-galactosidase activity. Bars represent 200 µm.

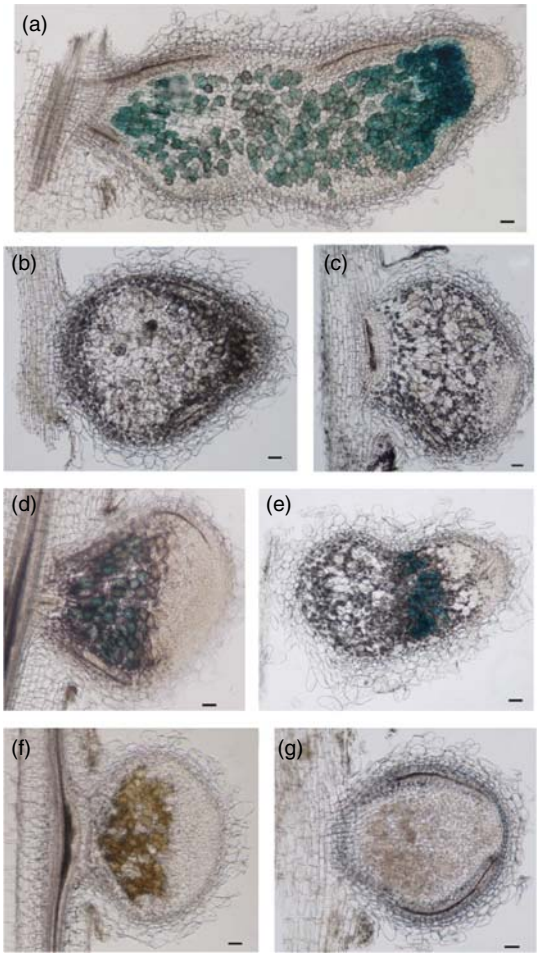

Figure 63.4 Expression of the *nifH*::*uidA* rhizobial marker gene inside mutant plant nodules. Nodules were harvested 3 weeks postinoculation with *S. meliloti strain* CSB357, and the activity of *nifH* promoter–uidA fusions was visualized with GUS staining in nodules developed on wild-type (a), 13U (b), *dnf8* (c), 5L (d), 11S (e), 7Y (f), and *dnf5-2* (g) roots. Bars represent 100 µm.

The formation of spherical and elongated nodules and the impaired bacterial colonization of the *ipd3/dnf5* double mutants were similar to the single *ipd3-1* plants, indicating that *IPD3* is epistatic to *DNF5* (Fig. 63.3).

The defects in symbiotic nitrogen fixation were also validated by analyzing the activity of the bacterial *nifH* promoter fused with the *uidA* (β-glucuronidase) gene. After 18 days inoculation with *S. meliloti* containing the P*nifH::uidA* fusion, wild-type *M. truncatula* nodules displayed high level of *nifH* expression in the different nodule zones (Fig. 63.4a). 5L and 11S nodules displayed a low level of *nifH* expression in the indeterminate zone (Fig. 63.4d, e), indicating that the arrest of the symbiotic interaction prior to invaded nodules cells is initiated in the nitrogen fixation zone. The mutants 7Y, 12AA, 13U, and 14S were all defective in inducing the *nifH* activity which correlates with the low occupancy of nodules by rhizobia in these mutants (Fig. 63.4b, c, f, g).

The effective functioning of the symbiotic nodules is accompanied by the simultaneous morphological differentiation of the nodule cells and rhizobia (Maunoury et al., 2010). To investigate the differentiation of rhizobia in the Fix- mutants, the bacterial morphology was analyzed following staining with the nucleic acid-binding dye SYTO13 and observed using confocal laser scanning microscopy (Haynes et al., 2004). Elongated bacteria could be observed in the nodule cells of wild-type plants (Fig. 63.5a). In contrast, only plant nuclei and the IT fluoresced in the *ipd3-1* elongated-type nodules (Fig. 63.5b) (Horvath et al., 2011), indicating that nodule cells were not invaded by rhizobia but were retained inside the IT. Most IT displayed an abnormal morphology; they were thick and highly crooked with enlarged, blister-like formations (Horvath et al., 2011). From these analyses, we conclude that the major function of *IPD3* is to control bacterial release from the IT. The nodules of *dnf5-2* contained nonelongated rod-shaped bacteria, indicating the failure of bacteroid differentiation

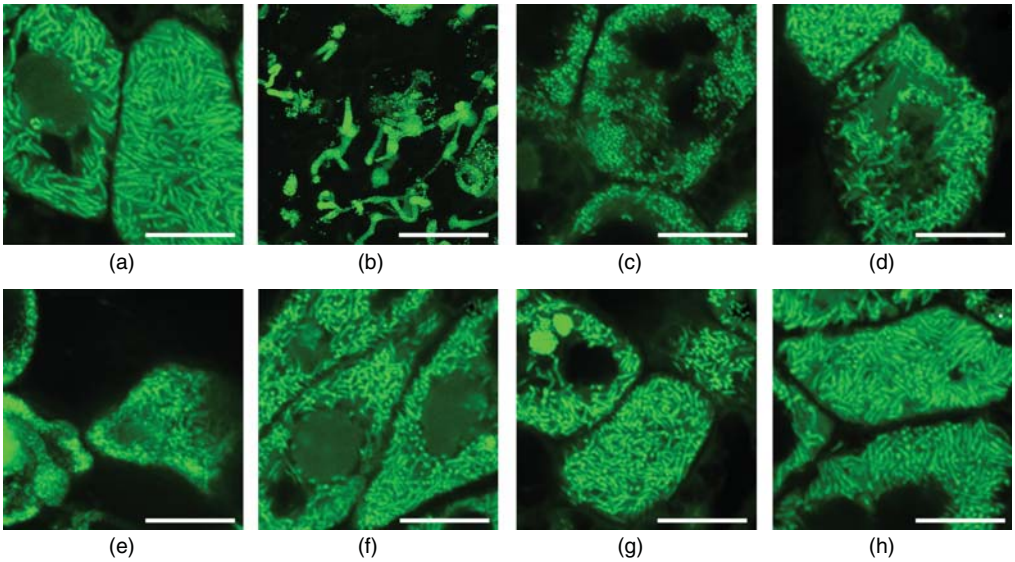

Figure 63.5 Bacterial morphology in *M. truncatula* wild-type (a) and ineffective nodulation mutant nodules (b–h). Longitudinal sections of nodules 4 wpi with *S. meliloti* were stained with SYTO13 and analyzed by confocal microscopy. The analysis revealed elongated bacteroids in the infected cells of wt nodules in the nitrogen fixation zone (a). The elongated nodules of *ipd3-1* did not contain released bacteria, but plant nuclei and infection threads with retained undifferentiated bacteria were detected (b). No bacterial elongation was observed in *dnf5-2* (c) nodule cells, indicating defect in bacterial differentiation. The bacterial development is initiated in the nodules of the other ineffective mutants (*dnf8* (d), 7Y (e), 5L (f), 11S (g), and 13U (h)). Bars represent 20 μm.

(Fig. 63.5c). The 5L and 11S nodules are dotted with a few cells containing bacteria in the nitrogen fixation zone (Fig. 63.2e, f), and these cells harbored rhizobia presenting limited bacteroid differentiation (Fig. 63.5f, g). 7Y and *dnf8* nodules did not contain infected cells in the nitrogen fixation zone (Fig. 63.2d, g), but elongated and branched bacteroids could be detected in the interzone cells (Fig. 63.5d, e). The few invaded cells of 13U nodules (Fig. 63.2h) contained elongated bacteria (Fig. 63.5h), indicating that the impaired gene is required for the persistence of rhizobia in the infected nodule cells rather than for bacterial differentiation.

63.3.4 Expression Pattern of Symbiotic Marker Genes Differentiates between Ineffective Mutants

The nodule formation and the initiation of nitrogen fixation are a result of a complex developmental program accompanied by transcriptional changes in both symbiotic partners (Maunoury et al., 2010; Moreau et al., 2011). Hybridization techniques and *in silico* analyses revealed several differentially expressed late nodulin genes (Mitra and Long, 2004; Starker et al., 2006; Maunoury et al., 2010; Moreau et al., 2011) which can be used as markers to dissect the *M. truncatula*–rhizobial symbiotic interaction. In order to differentiate between the progressions of the ineffective mutants isolated in this study at the transcription level, we selected ten symbiosis-specific genes (*MtLEC4*, *MtLb1*, *MtCAM1*, *MtN31*, *MtCP2*, *MtIPD3*, *MtNOD25*, *MtNOD26*, *MtNAP2*, and *MtNCR121*). We monitored rhizobia-induced expression of these symbiotic marker genes 14 days following bacterial inoculation (dpi) using quantitative RT-PCR (Fig. 63.6).

The nodule-specific *MtIPD3* gene, the member of the symbiosis signaling pathway, is required for rhizobial and mycorrhizal colonization (Horvath et al., 2011; Ovchinnikova et al., 2011), and following a transient decrease between 0 and 5 dpi, it is strongly expressed in wild-type nodules 15 dpi (Messinese et al., 2007). Based on the nodulation phenotype of the *ipd3* mutants (Figs. 63.1 and 63.2) (Horvath et al., 2011), we assumed that the functioning of the IPD3 protein precedes all the genes impaired in the ineffective nodulation mutants of this study. According to this, similar or moderate reduction of *IPD3* expression was detected compared to wild type in the ineffective nodulation mutants (Fig. 63.6).

Corresponding to the early arrest of the symbiotic process in *ipd3-1*, *dnf5-2*, and 7Y (Fig. 63.2), transcriptional activation of all the symbiotic marker genes was blocked or severely reduced in these mutants (Fig. 63.6). One interesting exception however was *NCR121* which was induced in *dnf5-2* to levels comparable to wild-type plants, but which was strongly reduced in *ipd3-1* and 7Y. The expression of *MtNAP2*, encoding a nodule-specific protein with unknown function, was reduced in all ineffective mutants compared

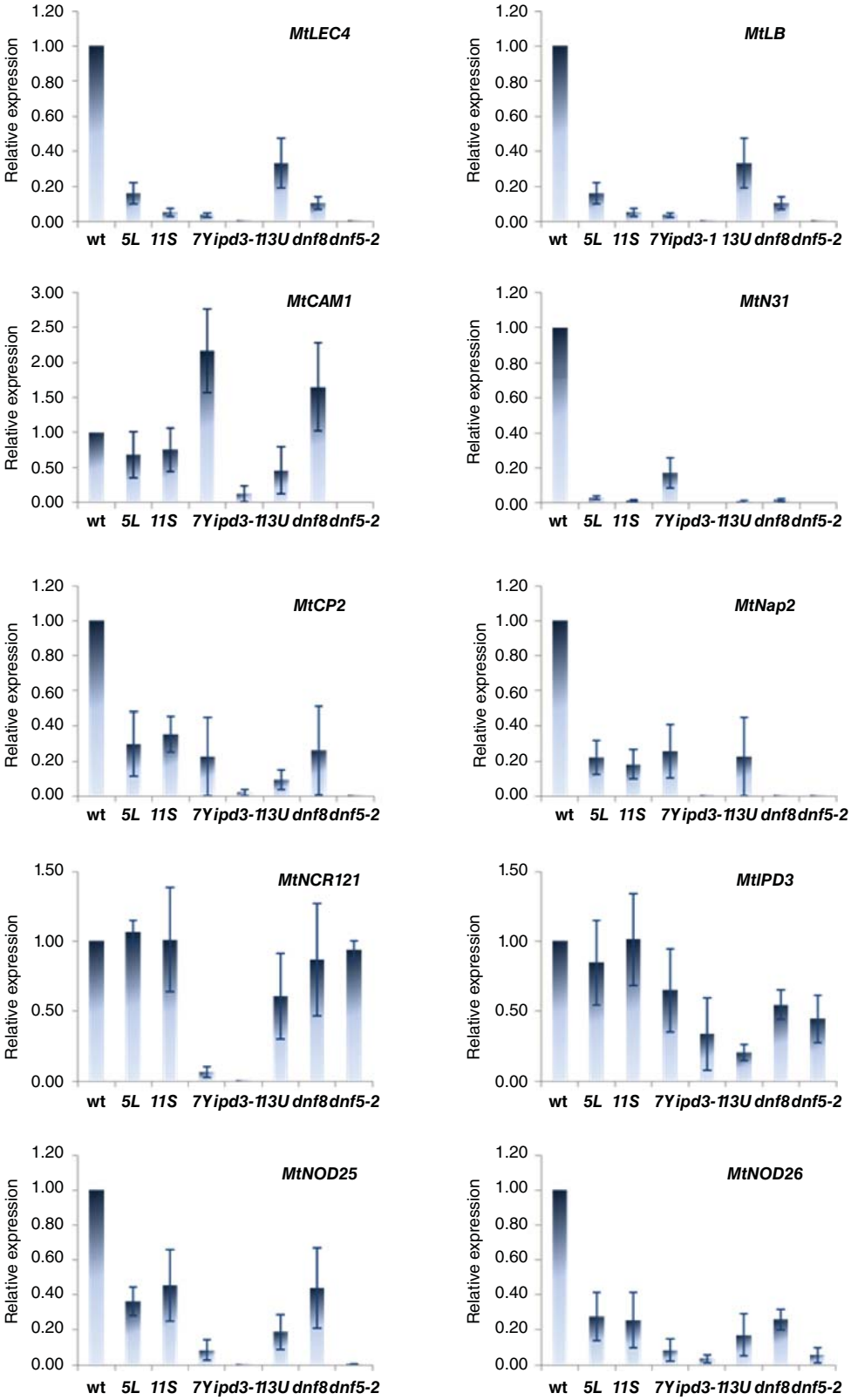

Figure 63.6 Expression analysis of selected nodule-specific genes in *M. truncatula* ineffective mutants 14 days after inoculation with *S. meliloti* 1021. The expression of genes *MtLEC4*, *MtLB*, *MtCAM1*, *MtN31*, *MtCP2*, *MtNAP2*, *MtNCR121*, *MtIPD3*, *MtNOD25*, and MtNOD26 was analyzed relative to wild type using RT-PCR. Three biological replicates for each mutant with three technical repeats were used for the analysis. Error bars represent SE.

to wild-type plants (Fig. 63.6). *MtNAP2* was expressed at lower level in nodules containing some infected cells in the nitrogen fixation zone, but the complete failure of its induction that was detected in *ipd3-1*, *dnf5-2*, and *dnf8* is related to the absence of nodule zonation or lack of bacterial occupancy in the nitrogen fixation zone (Figs. 63.3 and 63.6). The *MtLB1*, *MtNOD25*, and *MtNOD26* late nodulins were expressed at lower level in the ineffective mutants of this study than in wild type, and they were not or hardly induced in the early ineffective mutants *ipd3-1* and *dnf5-2*. One of the marker genes of early senescence in nodules, the cysteine protease gene *MtCP*, was expressed differently in the ineffective symbiotic mutants (Fig. 63.6). The *MtN31* transcript, encoding a nodule-specific Cys-rich protein (*NCR158*), could be detected at very low level in all ineffective mutants compared to wild type, indicating that its expression accompanies functioning symbiotic nodules (Fig. 63.6). The expression data of the symbiotic marker genes were consistent with the block of the symbiotic nodule development detected by microscopic analysis.

63.3.5 IPD3 Is Required for DMI3-Induced Spontaneous Nodule Formation Irrespective of the Genetic Background

The characterization of the mutant phenotypes and the analysis of gene expression indicated that *IPD3* appears to be the earliest acting gene of the genes impaired in the seven ineffective mutants. The gene was cloned by positional cloning (Horvath et al., 2011; Ovchinnikova et al., 2011), and it was demonstrated that it encodes the interacting protein of DMI3 (IPD3) (Messinese et al., 2007), the ortholog of *L. japonicus* CYCLOPS (Yano et al., 2008; see Chapter 59). Detailed phenotypic characterization and molecular studies revealed that IPD3 is required for rhizobial and mycorrhizal colonization of the host plant and is necessary for appropriate NF-induced gene expression (Horvath et al., 2011; Ovchinnikova et al., 2011). These data indicated that IPD3 is a member of the common Sym pathway. However, differences in the severity of *ipd3* mutants could be observed depending on the genetic background (Horvath et al., 2011).

It was demonstrated earlier that the autoactive form of DMI3 (DMI3 1–311*) could not induce spontaneous nodule formation, but the gain-of-function version of the cytokinin receptor MtCRE1 did trigger spontaneous nodules on *ipd3-3* roots (Ovchinnikova et al., 2011). In order to analyze whether the ability of spontaneous nodule formation depends on the genetic background, the gain-of-function version of DMI3 (DMI3 1–311*) (Gleason et al., 2006) was introduced into *ipd3-1* (*M. truncatula* Jemalong background) and *ipd3-2* (*M. truncatula* ssp. *tricycla* R108 background) roots. Spontaneous nodule formation was observed on *dmi3* roots (52 nodules/36 transformed roots) (Fig. 63.7a–c), and several

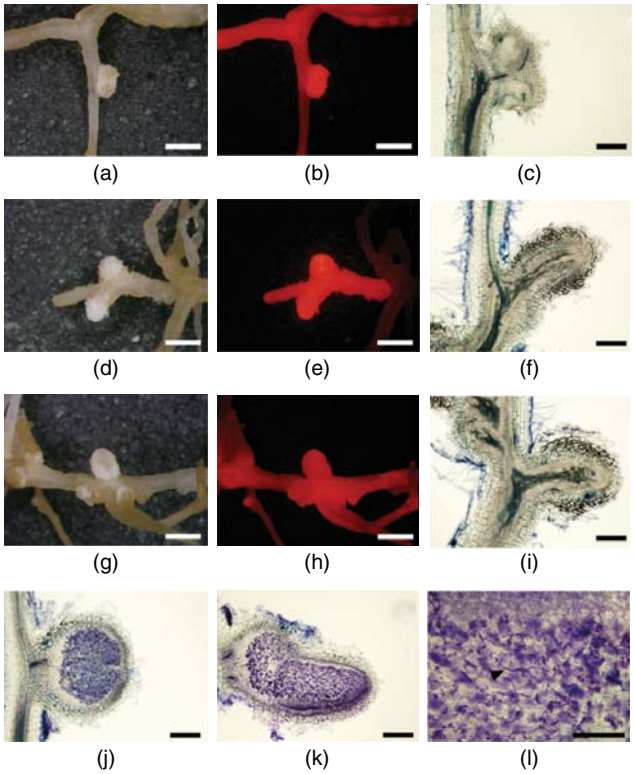

Figure 63.7 Testing the ability of spontaneous nodule formation on *dmi3* and *ipd3* mutants roots expressing the autoactive form of DMI3 (35S::DMI3 1–311*). 70 µm sections of nodule and root structures were prepared and stained with toluidine blue. Spontaneous nodule formed on *dmi3* roots (a–c), and only emerging lateral roots were detected on *ipd3-1* (d–f) and ipd3-2 (g–i) roots. The toluidine blue staining of spherical (j) and elongated (k) *ipd3-1* nodules induced by *S. meliloti* infection on native roots revealed extended invasion zones with hypertrophied infection threads with retained bacteria (l). Scale bars: 1 mm in (a), (b), (d), (e), (g), and (h); 200 µm in (c), (f), (i), (j), and (k); and 50 µm in (l).

nodule-like structures formed on *ipd3* roots (Fig. 63.7d–i). The microscopic analysis of the sections of these outgrowths showed that these structures had central vascular systems which indicated that they were emerging lateral roots and not spontaneous nodules. In addition, toluidine-blue staining did not detect bacteria entrapped in infection threads in these structures which is characteristic for nodules developed on native roots of *ipd3* (Fig. 63.7j–l). Based on these data, we could conclude that no spontaneous nodule formation was induced on *ipd3-1* (49 transformed roots) and *ipd3-2* (24 transformed roots) mutant roots, indicating no differences in the ability of spontaneous nodule formation between *ipd3* mutants in different genetic backgrounds.

63.4 DISCUSSION

Symbiotic nitrogen fixation mutants proved to be powerful and useful tools to dissect the nitrogen-fixing symbiotic

interaction and identify several essential components of the Sym signaling pathway (Venkateshwaran et al., 2013; Oldroyd, 2013; see Chapters 42, 55, 59, 110). Despite the identification of few genes required for bacterial release, persistence of bacteria, and nodule function in *M. truncatula* (Wang et al., 2010; Horvath et al., 2011; Ovchinnikova et al., 2011; Bourcy et al., 2013), relatively little is known about genes necessary for nodule invasion and maintenance of rhizobial symbiotic interaction. Early genetic screens carried out on EMS- or FN-mutagenized *M. truncatula* populations identified several ineffective nodulation mutants (Benaben et al., 1995; Starker et al., 2006; Teillet et al., 2008). Large-scale genetic screens have been initiated recently to identify mutants defective in nodule function (Pislariu et al., 2012; http://medicago-mutant.noble.org/mutant/FNB.php; see Chapter 83). These screens were carried out on FN-mutagenized *M. truncatula* Jemalong and retrotransposon-insertion *M. truncatula* ssp. *tricycla* R108 populations. In order to enlarge the collection of mutants impaired in the later stages of the *M. truncatula–S. meliloti* symbiotic interaction which fail to support efficient nitrogen fixation, a genetic screen of FN- and chemically (EMS) mutagenized *M. truncatula* Jemalong plants was carried out, as described here Domonkos et al. (2013). The seven ineffective mutants analyzed in this study represent six complementation groups. Although an incomplete allelism test has been carried out between the mutants isolated in this study and previously identified complementation groups, two novel loci (*ipd3-1* and *dnf8*), a new allele of *dnf5*, and probably three new complementation groups could be identified which indicates that the collection of ineffective *M. truncatula* mutants is far from saturation.

The characterization of the mutant phenotypes and the analysis of gene expression presented here and the study of Domonkos et al. (2013) allowed us to define the functional hierarchy of the impaired genes we identified. *IPD3* (see Chapter 59) and *DNF5* appear to the earliest acting genes, with both severe nodulation defects and greatly reduced symbiotic gene expression. However, the degree of bacterial invasion, the expression of *NCR121*, and the analysis of the epistatic relationship using double mutants showed that *DNF5* acts later in the symbiotic process than *IPD3*. The disintegration of rhizobia and the sporadic brown pigmentation in the fixation zones of mutants 5L/11S and 13U show incompatible bacterial interactions and induction of early senescence. The genes impaired in mutants 5L/11S and 13U appear to be required for the maintenance of bacterial infection, particularly in the nitrogen fixation zone of the nodule. While *DNF8* has a similar nodulation defect, the gene profiling suggests it acts at a slightly later stage than mutants 5L/11S and 13U. The presence of bacteria in the early developmental zones of *dnf8* nodules and the complete lack of rhizobia in the nitrogen fixation zone probably indicate the arrest of rhizobial differentiation in this mutant. The strong brown pigmentation in the 7Y mutant may indicate misregulation of plant defenses. If this is correct, then positioning 7Y within this sequence of loci is questionable, since the timing of the gene function may not be directly related to the stage at which the mutant aborts. However, such a statement could be true for all the genetic loci described, and a role for *IPD3* (*CYCLOPS*) during symbiotic signaling (Oldroyd, 2013; Messinese et al., 2007; Singh and Parniske, 2012) belies its apparently late mutant phenotype (Horvath et al., 2011; Ovchinnikova et al., 2011).

The characterization of the ineffective mutants may suggest the possible function of the impaired genes, but in order to assess their actual function and characterize their gene products, these genes need to be cloned. Despite the great advantages of the insertion mutants that allow the recovery of the genomic sequences adjacent to the integration sites of the transposons (Pislariu et al., 2012), the genomic resources of *M. truncatula* also render the rapid identification of deleted genes by either map-based cloning or transcript-based cloning, as successfully demonstrated in several recent papers (Horvath et al., 2011; Murray et al., 2011). The cloning and molecular characterization of genes defective in the ineffective *M. truncatula* mutants of this study will provide more details about the process of nodule invasion and differentiation and contribute to a better understanding of the molecular, developmental, and differentiation events that support a nitrogen-fixing nodule.

IPD3 is closely homologous to *L. japonicus CYCLOPS* (Yano et al. 2008), and *CYCLOPS* can complement the *ipd3-1* mutant (Horvath et al., 2011; see Chapters 54, 59). Despite their orthologous relationship, the *ipd3* and *cyclops* mutants show differences in the severity of the phenotype (Horvath et al., 2011; Yano et al., 2008). In addition, phenotypic difference in the symbiotic interaction with rhizobia and AM fungi could be also observed between *M. truncatula* plants with different genetic backgrounds. *ipd3* mutants (*ipd3-1*, *ipd3-3* = *Mtsym1-1* and *Mtsym1-8*) in the Jemalong background develop two classes of nodules; the majority of the nodules are small spherical white structures with abnormal morphology at the nodule apex (Fig. 63.2b), and a small number of nodules are less or remarkably elongated containing extended infection zone woven into the network of IT (Horvath et al., 2011; Benaben et al., 1995; Ovchinnikova et al., 2011; E. Limpens, personal communication). Bacterial release could never be detected in these nodules. Contrary, only the induction of nodule primordia could be observed on roots of *ipd3-2* plant with R108 (*M. truncatula* ssp. *tricycla* R108) background. The severity of the mycorrhizal defect also appears to be dependent on the genomic background in *ipd3* mutants. *ipd3-1* and *ipd3-3* alleles (Jemalong background) developed normal internal fungal infection structures, with apparently similar to wild-type arbuscules, but at a reduced frequency (Horvath et al., 2011; Ovchinnikova et al., 2011). In contrast, the *ipd3-2* allele

Table 63.3 The Summary of *ipd3*/*cyclops* Phenotypes in Different Legume Backgrounds

	Mutant					
	Mtipd3-1	*Mtipd3-2*	*Mtsym1-1* (TE7)	*Mtsym1-8*	*Ljcyclops*	*Pssym33*
Genetic background	Jemalong	Tnt1-insertion line in R108	Jemalong	*Tnt1*/Mere insertion line in Jemalong	Ecotype Gifu	SGE
Infection and nodulation phenotype	Two classes of nodules	Nodule Primordia	Two classes of nodules	Similar phenotype as *Mtsym* – E. Limpens, personal communication	Nodule primordia	White nodules; impaired invasion (no endocytosis) (Voroshilova et al., 2001)
AM phenotype	Arbuscule formation; reduced colonization rate	No arbuscules	Decrease colonization but abundance of arbuscules	?	No arbuscules	Decreased AM colonization but increased arbuscule abundance (Jacobi et al., 2003a, 2003b)

(R108 background) shows a complete absence of arbuscules. Interestingly, the reported phenotypes of orthologous *ipd3* alleles in other legumes also display distinct symbiotic phenotypes (Table 63.3). The symbiotic phenotype of *L. japonicus cyclops* mutants is very similar to the *M. truncatula ipd3-2* (R108) mutant, although nodule organogenesis is initiated in *Ljcyclops* but blocked in very early stage (Yano et al., 2008). The rhizobial and AM infections abort in *cyclops* prematurely: IT growth could not be observed, and intracellular AM infection is severely impaired. However, the phenotype of pea *ipd3* mutant (*Pssym33*) resembles the phenotype of *M. truncatula* Jemalong mutants (Ovchinnikova et al., 2011). White nodules are formed on *Pssym33* roots wherein the rhizobial invasion is impaired (Voroshilova et al., 2001) and decreased mycorrhizal colonization was observed with abundance of arbuscules (Jacobi et al., 2003a, 2003b). The cause of the phenotypic differences between different *ipd3*/*cyclops* mutants still remains to be disclosed. Using the autoactive form of CCaMK (DMI3), it was found that CYCLOPS is not required for nodule organogenesis beyond the primordial stage (Yano et al., 2008). However, spontaneous nodulation test revealed that IPD3 affects DMI3-induced spontaneous nodule formation. Using the autoactive form of DMI3, we could not detect difference in the ability of spontaneous nodulation in the *Mtipd3* alleles in different genetic backgrounds, indicating that *ipd3-2* is similar to *Ljcyclops* with respect to the ability to form spontaneous nodule.

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Chapter 64

Multifaceted Roles of Nitric Oxide in Legume–Rhizobium Symbioses

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64.1 INTRODUCTION

64.1.1 NO: A Key Molecule

NO is a small reactive gaseous molecule which, as a mediator of physiological processes, has a large number of beneficial effects in animals (messenger in immune response, neurotransmission, and relaxation of vascular smooth muscle) (Schmidt and Walter, 1994). The cellular environment may greatly influence the chemical form of NO and consequently its biochemical and biological effects. Not only the chemical nature but also the concentration and location of NO might influence its biological role. Thus, at high local concentration, such as on sites of its synthesis, NO can become very toxic. NO has also a vital role in the resistance of mammalian hosts to microbial infections as first established with murine macrophages colonized with *Mycobacterium bovis* (Flesch and Kaufmann, 1991). The interest for NO in the plant scientific community is more recent, but considerable progress has been made in the past decade in understanding the roles of NO in plants. The emerging picture is that NO functions as a signal involved in diverse physiological processes that include germination, root growth, lateral root development, flowering, stomatal closing, and adaptative responses to biotic and abiotic stresses (Baudouin, 2011; Besson-Bard et al., 2008; Delledonne, 2005; Gaupels et al., 2011; Mur et al., 2013). The molecular identification of NO sources and their subcellular characterization, together with specific and accurate NO detection methods, are limiting steps for understanding NO functions in plants.

Unlike in animal cells where NO is known to be mainly synthesized from arginine by NO synthase (NOS) enzymes, the origin of NO in plants is not completely understood yet.

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It is becoming increasingly obvious that nitrate reductases (NR) play a major role in NO production, but several studies provide evidence arguing for the involvement of NOS-like enzymes and nonenzymatic reduction of nitrite in NO synthesis. Alternately, NO could also be produced from polyamines through an uncharacterized mechanism (for a review, see Moreau et al. (2011)).

64.1.2 NO: A Signal/Weapon in Plant Defense

A rapid NO burst has been shown to be induced during plant–pathogen interactions, suggesting the involvement of NO in disease resistance (Mur et al., 2006; Wendehenne et al., 2004). Indeed, NO has been implicated in defense against various pathogenic organisms (*Pseudomonas syringae*, *Botrytis cinerea*, or *Blumeria graminis*) (Delledonne et al., 1998; Mur et al., 2012; Prats et al., 2005; Bellin et al., 2013). Considering these plant responses, it is not surprising that many pathogens have evolved responses that could suppress NO-associated events. Thus, *Erwinia chrysanthemi* expresses the flavohemoglobin (fhb) HmpX which oxidizes NO to NO_3^-. Conversely, in other cases, the pathogen may actively trigger host NO production to sustain the infection process (Lamotte et al., 2004; Prats et al., 2008).

The mechanisms of NO action are being actively investigated. The first studies concerned transcriptomic assessment of NO effects, but, although the action of NO on gene expression is still being investigated, a lot of studies are now dedicated to two protein posttranslational modifications which are specific to NO action/signaling, that is, S-nitrosylation and tyrosine nitration. There have been relatively few studies on tyrosine nitration possibly because its irreversible nature made it a less attractive regulatory switch, although increasing evidence of enzymatic and nonenzymatic mechanisms of denitration is available (Abello et al., 2009). On the contrary, S-nitrosylation, consisting of the reversible covalent attachment of a NO moiety to a protein or glutathione cysteine thiol, has received much more attention. About 15 S-nitrosylated plant proteins have been well characterized, many of them being involved in plant immunity such as NPR1, the salicylic acid-binding protein (SABP3), and the transcription factor TGA1, which are key proteins in the signal transduction pathway that leads to plant resistance against pathogens, drawing a first outline of the involvement of NO in plant immune signaling (Astier et al., 2012; Astier and Lindermayr, 2012).

64.1.3 NO: A Molecule Detected at All Stages of Rhizobium–Legume Symbioses

NO has also been detected during the symbiotic interaction between legumes and rhizobia. These bacteria interact with legume roots on which they induce the formation of new organs, called nodules. The bacteria colonize the nodules wherein they differentiate into bacteroids able to reduce atmospheric nitrogen (N_2) into ammonia (NH_3) to the benefit of the host plant. In exchange, plants provide bacteria with a niche (the nodule) as well as energy in the form of carbon sources. Rhizobium–legume symbioses have been thoroughly characterized at the cytological and genetic levels, and a number of bacterial and plant genes required for bacterial infection, nodule formation, and functioning have been identified (Jones et al., 2007; Oldroyd et al., 2011; Terpolilli et al., 2012; see also Chapter 59). Nodules can be of determinate (*Lotus japonicus*, *Glycine max* (soybean)) or indeterminate (*Medicago*) type. Indeterminate nodules have a persistent meristem that yields a cylindrical shape to the nodule where four different zones can be distinguished, the zone III being the N_2 fixation zone where bacteroids convert N_2 into NH_3. In contrast, determinate nodules lack a persistent meristem and are spherical.

NO is present at various stages of rhizobium–legume symbioses (Fig. 64.1). First, using a NO-specific fluorescent probe, a transient NO production was detected on the surface of *Medicago sativa*, *L. japonicus*, and *Trifolium repens* roots 4 h after inoculation but only when the cognate bacterial symbionts were used (Nagata et al., 2008; Shimoda et al., 2005) (see Fig. 64.1a). NO was also present around 4 days postinoculation in infected *Medicago truncatula* root hairs, both in the colonized curl and along the infection thread, as well as in cells of nodule primordia, not yet infected by rhizobia (del Giudice et al., 2011) (see Fig. 64.1b, c). In addition, NO was observed (by using either a fluorescent probe or a *Sinorhizobium meliloti* NO-biosensor strain) in mature, nitrogen-fixing nodules of *M. truncatula* and *M. sativa* particularly in bacteroid-containing cells (Baudouin et al., 2006; Pii et al., 2007) (see Fig. 64.1d, e). Finally, NO was also detected directly in mature nodules of *L. japonicus* (Shimoda et al., 2009), as well as indirectly, through the detection of nitroso-leghemoglobin in nodules of soybean and pea (Kanayama et al., 1990; Meakin et al., 2007). NR has been shown to be an important source of NO in nodules (Horchani et al., 2011), but its involvement and that of other possible NO sources at the different steps of the symbiosis remain to be clarified. Interestingly, both partners (bacteria and plant) were shown to participate significantly in NO synthesis in determinate (soybean) as well as indeterminate (*Medicago*) nodules (Horchani et al., 2011; Sanchez et al., 2010).

The purposes of this review are to highlight the contrasted roles that NO might have at the different steps of symbiosis and to point out the importance not only of plant hemoglobins (Hbs) but also of bacterial NO-degrading proteins in maintaining a balanced NO level allowing the maintenance or breakdown of symbiosis.

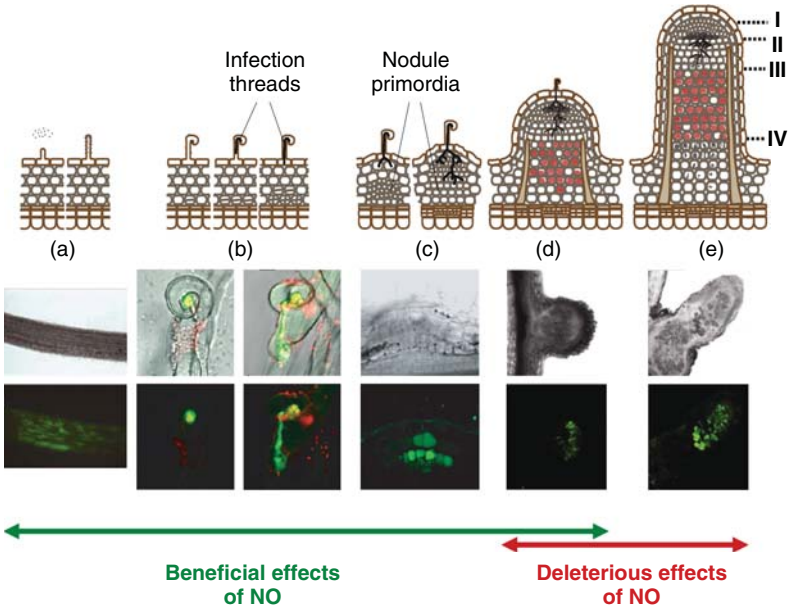

Figure 64.1 NO detection at various stages of the *Sinorhizobium–Medicago* interaction. The upper part of the figure illustrates the successive stages of the symbiotic interaction where NO was found, as shown in the lower part of the figure. I, II, III, and IV correspond to the meristematic, infection, fixation, and senescence zones of mature indeterminate-type nodules, respectively. NO was detected by microscopy using the fluorescent probe DAF-2DA on either *M. sativa* (a) or *M. truncatula* (b–e) roots or nodules, collected at 4 h (a), 3 days (b, c), 5 days (d), and 19 days postinoculation with *S. meliloti* (e). *Source*: Drawings and pictures composing this figure were reprinted with modifications from the following references, with kind permissions from the publishers and corresponding authors: Fig. 1 in (Saeki, 2011), Springer Science + Business Media; Fig. 5 in (Nagata et al., 2008), APS Press; Fig. 1 and Fig. 4 in (del Giudice et al., 2011), Wiley-Blackwell; Fig. 1 in (Baudouin et al., 2006), APS Press.

64.2 NO: POSITIVE/SIGNALING ROLES ON SYMBIOSIS ESTABLISHMENT AND NODULE FUNCTIONING

64.2.1 Infection and Nodule Development

The production of NO along the infection process points to a role of this compound in the specific recognition between the plant and the bacterial partners. In the *M. truncatula–S. meliloti* interaction, both the scavenging of NO by 2-[4-carboxyphenyl]-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (cPTIO) and the overexpression by the plant partner of *hmp*, a bacterial hemoprotein involved in NO detoxification (Poole and Hughes, 2000), lead to a delayed nodulation phenotype (del Giudice et al., 2011). This indicates that NO is required for an optimal establishment of the symbiotic interaction and nodule development. A first transcriptomic study conducted on *M. truncatula* roots treated with two NO donors, SNP and GSNO, enabled the identification of 999 putative NO-responsive genes (Ferrarini et al., 2008). Of these genes, 290 were also regulated during nodule development. More recently, a transcriptomic analysis using RNA-Seq technology was performed with *M. truncatula*-inoculated roots treated with cPTIO to identify genes potentially regulated by NO during the nodule primordium development (Boscari et al., 2013). The first result of this study shows the reversal, upon cPTIO treatment, of the downregulation of defense genes normally triggered by inoculation with rhizobia. Expression of a number of genes involved in terpene, flavonoid, and phenylpropanoid pathways and genes encoding PR proteins and cytochrome P450 is significantly affected by cPTIO. In contrast, cPTIO triggers the repression of genes potentially involved in nodule development such as those involved in regulation of transcription, protein transport, degradation, and synthesis. These findings suggest that NO could be involved in the repression of plant defense reactions, thereby favoring the establishment of the beneficial plant–microbe interaction. This action differs markedly from the signaling functions of NO in pathogenic interactions, in which NO induces hypersensitive cell death, and expression of defense-related genes (Delledonne et al., 1998).

In both transcriptomic studies (Boscari et al., 2013; Ferrarini et al., 2008), many genes involved in the developmental program of root hairs during nodulation (kinases, receptor-like kinases, transcription factors), in carbon metabolism (sucrose transport, sucrose synthase, isocitrate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, or malate dehydrogenase), as well as in proteasome-dependent proteolysis were found to be upregulated by NO, indicating that early nodule establishment and metabolism are controlled by NO. Many genes involved in the control of cellular redox responses such as peroxidases, germin-like oxalate oxidase, glutathione S-transferase, and glutathione synthetase (GSHS) have also been identified. A previous study on the two genes involved in glutathione synthesis, γ-glutamylcysteine synthetase (γ-ECS) and GSHS, already provides evidence that glutathione synthesis is stimulated by NO in plants (Innocenti et al., 2007). These data indicate that NO-mediated redox signaling plays a key role in the establishment of a functioning nodule, as it was shown to be the case for glutathione-mediated regulation of the symbiosis (Frendo et al., 2005). It was also suggested that,

in the rhizobium–legume symbiosis, early production of NO by the plant could serve to induce the expression of bacterial genes necessary to adapt bacteria to NO encountered at later symbiotic stages (Meilhoc et al., 2011). The lower competitiveness of the *hmp*$^{++}$ strain as compared to the wild type (WT) during the *M. truncatula–S. meliloti* interaction (del Giudice et al., 2011) is an argument in favor of such a role of NO during the infection process. Regarding to the relationship between NO and oxidative stress, it was shown that during the symbiotic association between fungi and lichens, the mycobiont-driven NO has an important role in the regulation of the oxidative stress of lichens, particularly during rehydration (Catala et al., 2010). A similar role could be conceived in the rhizobium–legume symbiotic interaction where an oxidative burst occurs early after infection with symbiotic bacteria (Santos et al., 2001).

Among the NO-responsive genes in early legume–rhizobium interactions, the class 1 non-symbiotic hemoglobin (*ns-Hb1*) gene is of particular importance. A NO-scavenging activity has been associated with ns-Hb1 in NO detoxification pathways (Gupta et al., 2011). In *M. truncatula*, a decrease in the transcript level of *ns-Hb1* at four days postinoculation has been observed, suggesting that a decrease in ns-Hb1 level could allow an increase in the NO level in the root during the establishment of the symbiotic interaction (Boscari et al., unpublished). Conversely, the overexpression of *ns-Hb1* genes *LjnHb1* or *AfnHb1* in *L. japonicus* induces a decrease in NO production and results in an increased nodule number (Shimoda et al., 2009), suggesting that NO negatively affects the nodulation process. The apparent contradiction in nodulation phenotypes between *M. truncatula* (del Giudice et al., 2011; Pii et al., 2007) and *L. japonicus* (Shimoda et al., 2009) raises the question of the effect of Hmp/Hb on the NO level and suggests that a tightly regulated level of NO is necessary for successful establishment of the symbiotic relationship.

During the early steps of symbiosis establishment, NO was also detected in dividing cortical cells of the root, not yet invaded by the rhizobial cells (del Giudice et al., 2011) (see Fig. 64.1c). This NO production is similar to that detected in lateral root primordia for which NO was reported to have a role in the cell cycle regulation and in the control of primary and adventitious root organogenesis (Correa-Aragunde et al., 2004; Correa-Aragunde et al., 2006; Lanteri et al., 2006). In *M. truncatula*, NO scavenging resulting either from cPTIO treatment or from overexpression of a bacterial flavohemoglobin *hmp* gene has been found to downregulate several genes involved in nodule development, such as *MtCRE1* and *MtCCS52A* (del Giudice et al., 2011). *MtCRE1* encodes a cytokinin receptor playing a major role in the development of the symbiotic interaction between *M. truncatula* and *S. meliloti* (del Giudice et al., 2011; Gonzalez-Rizzo et al., 2006; see Chapter 56). As a consequence of NO decrease, delayed nodulation and reduced expression of genes involved in nodule development (*MtCRE1*, *MtCCS52A*) reinforce the hypothesis that NO could control the nodulation process through the regulation of cytokinin perception (del Giudice et al., 2011). NO has already been described as a component of hormone signaling in plants (Hill, 2012). Phytohormones such as auxin or abscisic acid (ABA) are known to regulate nodulation (see Chapter 56). Interestingly, the work of Pii et al. (2007) showed that auxin overexpression in *Medicago* species increased nodulation (number of nodules/plant) and NO level in these nodules (Pii et al., 2007). On the other hand, the level of ABA, which is known to be a negative regulator of legume root nodule formation, is diminished in an *enf1* mutant of *L. japonicus* where NO level is diminished accordingly, together with an increase in nitrogen fixation (Tominaga et al., 2009). The precise roles of NO in these hormonal pathways remain to be deciphered.

As a whole, NO appears to be a key regulator of symbiotic establishment, acting as a repressor of the plant defense responses when the bacteria enter into the root and an inducer of organogenesis and development processes which lead to nodule formation.

64.2.2 Mature Nodule Functioning

The role of NO in mature nodules is still an open debate. Indeed, NO has been shown to be significantly produced in the fixation zone of *M. truncatula* nodules (Baudouin et al., 2006) (see Fig. 64.1d), and the question was raised of its potential role in the N_2-fixing nodules. Based on known adaptation mechanisms of plants to hypoxia and considering that nodules are microoxic organs, a metabolic role for NO has been recently proposed in mature nodules (Horchani et al., 2011). NO production has been shown to be induced in the roots of plants submitted to hypoxia, and this production is supposed to be linked – via a cyclic respiration process – with improved capacity of the plants to cope with hypoxic stress and to maintain cell energy status (Igamberdiev and Hill, 2009). This cyclic respiration, called "nitrate–NO respiration," involves four successive steps: (i) the reduction of NO_3^- to NO_2^- by the cytosolic NR; (ii) the translocation of NO_2^- from the cytosol into the mitochondria, presumably through a nitrite transporter; (iii) the reduction of NO_2^- in NO, via the mitochondrial electron transfer chains (ETC), allowing respiration and ATP regeneration; and (iv) the passive diffusion of NO from the matrix to the cytosol, where it is oxidized by Hb into NO_3^-. Thus, under hypoxic conditions, by reducing nitrite to NO, plant mitochondria preserve the capacity to oxidize external NADH and NADPH and retain a limited power for ATP synthesis complementing glycolytic ATP production (Gupta and Igamberdiev, 2011).

Under hypoxic conditions, NO production has been shown to be increased in functional nodules of *G. max*

(Meakin et al., 2007) and *M. truncatula* (Horchani et al., 2011), suggesting that, under the microaerobic conditions prevailing in nodules, nitrate–NO respiration may be involved in energy supply for nitrogen fixation (Horchani et al., 2011). Several observations argue in favor of such a hypothesis. First, plant NR and ETC, and the bacterial denitrification pathway contribute to NO production, via NO_3^- and NO_2^- reduction, particularly under hypoxic conditions (Horchani et al., 2011; Sanchez et al., 2010). Second, leghemoglobins (Lbs) and ns-Hb1 have the capacity to efficiently react with NO to produce NO_3^- with an elevated rate constant (Herold and Puppo, 2005), and the NO generated at the ETC level may therefore be oxidized into NO_3^- by Lbs and/or nonsymbiotic hemoglobins (ns-Hbs). Third, the energy status of the nodules depends either significantly or almost entirely on NR functioning under normoxic or hypoxic conditions, respectively (Horchani et al., 2011). Thus, in symbiotic nodules, a role related to NO scavenging and metabolism may be fulfilled by Hbs and Hmp in the plant and bacterial partner, respectively. The high affinity of these Hbs for NO and their capacity to oxidize NO into NO_3^- would be favorable to keep NO concentration below toxic level for nitrogenase and to supply the nitrate–NO respiratory cycle in order to maintain a minimal energy status under hypoxia. Considered together, these data suggest that in mature nodules, NO could function as a metabolic intermediate to maintain basal energy metabolism.

NO present inside nodules could also be perceived as a signal by bacteroids. Bacteria possess transcription factors able to sense NO and respond by adjusting bacterial gene expression to this changing environment. A transcriptomic analysis performed on *S. meliloti* grown in culture treated with a NO donor identified a set of about 100 genes whose expression is induced in the presence of NO (Meilhoc et al., 2010). Most of these genes are regulated by the two-component system FixLJ already known to respond to microaerobic conditions which prevail inside mature nodules. Indeed, FixLJ was shown to be a major regulator in symbiosis since it controls the majority of the rhizobial genes expressed *in planta* in mature nodules (Bobik et al., 2006). Hence, the NO response mediated by FixLJ comprises genes involved in microaerobic respiration and NO degradation (*hmp*) and many other genes of yet unknown function. Whether NO signaling via FixLJ has a biological significance in symbiosis remains to be established.

NnrR is a well known NO-specific responsive regulator in rhizobia (Rodionov et al., 2005). It has been shown to be involved in the regulation of a set of genes including the denitrification genes (*nir*, *nor*) in *S. meliloti* as well as in *Bradyrhizobium japonicum* and *Rhizobium etli* (de Bruijn et al., 2006; Cabrera et al., 2011; Meilhoc et al., 2011). It must be underlined that *nor* genes encode a NO reductase which has a role in degrading NO. Finally, we cannot exclude that the regulator OxyR denoted as an oxidative stress sensor could be also part of the NO signaling scheme in rhizobia. Indeed, OxyR has been shown to be S-nitrosylated in *E. coli* in the presence of NO, and in that form, it is involved in the regulation of expression of genes involved in monitoring the amount of intracellular S-nitrosylation (Gusarov and Nudler, 2012; Seth et al., 2012).

Hence, NO could be a signal to build up a bacterial NO response via different regulators, and the bacterial response could be involved in the establishment or maintenance of symbiosis in a more subtle way than the sole detoxification function.

64.2.3 NO and Nodule Senescence: Deleterious Effect or Signaling Role?

The concentration of NO inside nodules has not been measured precisely but was estimated to be in the micromolar range in *Medicago* nodules (Meilhoc et al., 2010). The NO level in nodules has even been shown to be significantly increased when nitrate was applied to *Lotus*, *Medicago*, or soybean nodules (Horchani et al., 2011; Kato et al., 2010; Sanchez et al., 2010). An inhibitory effect of NO ($Ki = 56\ \mu M$) has been first demonstrated employing the *B. japonicum* nitrogenase by using purified enzyme from soybean nodules and testing the effect of various NO concentrations *in vitro* (Trinchant and Rigaud, 1982). The effect of NO on nitrogenase *in vivo* has also been studied. However, as NO sources are not completely elucidated, pharmacological (exogenous NO or nitrate treatment) or genetic approaches (bacterial or plant mutants overexpressing NO-degrading enzymes) were used to manipulate NO levels inside nodules. Addition of a NO donor to *Lotus* nodules, although probably exceeding *in vivo* NO fluctuations in terms of concentration and duration, leads to a reduction in nitrogen fixation efficiency, as measured by the acetylene reduction assay (ARA test) (Kato et al., 2010; Shimoda et al., 2009). This effect has been confirmed indirectly in another study, where overexpression of the ns-Hb1 (NO-scavenging enzyme) in *L. japonicus* leads to an increased nitrogen fixation efficiency as measured on whole plants or on detached nodules (Shimoda et al., 2009). On the other hand, *M. truncatula* inoculated with a *S. meliloti* mutant (*hmp*) affected in NO degradation displays nodules presenting a higher NO content and a lower nitrogen fixation efficiency and shoot dry weight as compared to the WT strain (Cam et al., 2012).

The transcription level of the *nifH* gene (encoding the nitrogenase reductase subunit) of *Mesorhizobium loti* bacteroids has been shown to be similar in both WT and ns-Hb1-overexpressing *L. japonicus* nodules, indicating that the enhancement of nitrogen fixation efficiency is not due to a transcriptional effect of NO depletion (Shimoda et al., 2009). NO might also modify proteins through S-nitrosylation which emerges as a key posttranslational modification in plants and a pivotal mechanism to mediate

NO bioactivity. Nitrogenase displays at least three putative S-nitrosylation sites (Xue et al., 2010), and interestingly, different nitrogenase subunits have been identified among the S-nitrosylated proteins found in *M. truncatula* mature nodules (Puppo et al., 2013). These results suggest that NO may inhibit nitrogenase activity through S-nitrosylation; nevertheless, other major enzymes could also be NO targets. Thus, ammonium generated by bacterial nitrogenase activity is released by the bacteroids in the cytosol of infected plant cells where it is assimilated through the activity of the plant glutamine synthetase (GS). It has been shown recently that the *M. truncatula* cytosolic GS1 is modulated by another NO-mediated posttranslational protein modification: tyrosine nitration (Melo et al., 2011). According to the model proposed by the authors, NO present in nodules would contribute to a decrease in ammonium level (inhibition of nitrogenase) and to a coordinated inhibition of GS. It has also been proposed that inhibition of GS could be involved indirectly in antioxidant defense (Melo et al., 2011).

Among the S-nitrosylated proteins found in *M. truncatula* nodules, many are related to carbon- and nitrogen-fixing metabolism (Puppo et al., 2013), such as several enzymes of the tricarboxylic acid (TCA) cycle (isocitrate dehydrogenase, citrate synthase, malate dehydrogenase) from either the plant or the bacterial partner. A similar observation has already been made in pathogenic bacteria, such as *M. tuberculosis* or *Salmonella enterica* (Rhee et al., 2005; Richardson et al., 2011). Finally, NO may also target and inhibit bacterial respiration (Stevanin et al., 2000). The effect(s) of NO (inhibition or activation) remains to be determined as well as the role of most of these proteins and the impact of their S-nitrosylation in symbiosis.

Recently, it has been established that an increase in NO level inside mature nodules of *M. truncatula* leads to an early nodule senescence (Cam et al., 2012). From what we described earlier, this effect could be directly linked to an inhibition of nitrogenase activity and/or GS, leading to a cessation of nitrogen fixing and a subsequent collapse of bacteroids and plant cells. Although this cannot be completely ruled out, it has been found that 250 µM of the NO donor DEA-NONOate, when added to the plant roots, induced senescence without inhibiting nitrogenase activity. It has also been shown that NO could be a senescence signal acting locally rather than systemically (Cam et al., 2012). A NO burst preceding physiological nodule senescence could not be shown at the nodule scale, but interestingly, by using a *S. meliloti* NO-biosensor strain, an increase in NO level has been detected in a few cells in the center of the nodule. A correlation has been made with previous data from Perez Guerra et al. (2010) showing that the earliest signs of nodule senescence appear in a few infected cells in the center of the fixation zone. The senescence zone then extends with a conical front toward the nodule periphery (Perez Guerra et al., 2010). Considering this pattern, it has been hypothesized that NO accumulates in only a few cells before (and probably triggering) their entrance into the senescence process (see model in Fig. 64.2).

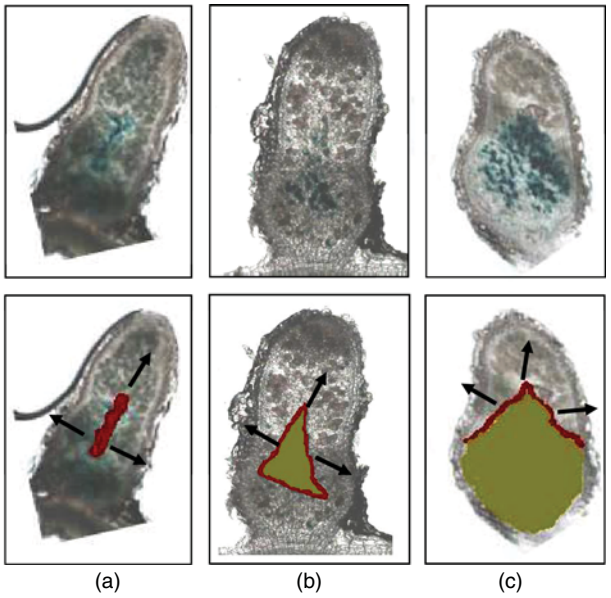

Figure 64.2 Proposed model for induction of nodule senescence by NO. Upper panels: NO detection in 3-week-old nodules using a NO-biosensor *S. meliloti* strain. This strain contains a reporter plasmid carrying a transcriptional *lacZ* fusion to the promoter of a *S. meliloti* gene specifically induced by NO. Reporter gene expression was detected using X-gal at different stages of senescence on sections of nodules containing the wild-type (a, b) or *hmp* mutant (c) strains. Lower panels: schematic representation of hypothetical NO burst starting in few cells of the center of the fixation zone (in red) and preceding the senescence zone which then extends with a conical front toward the nodule periphery (in green). Arrows indicate the direction of the senescence zone expansion. *Source*: Panels a and c were reprinted with modifications from Fig. 3 in (Cam et al., 2012), with kind permission from Wiley-Blackwell.

Lastly, overexpression of Hmp in WT nodules leads to a significant delay in nodule senescence which substantiates the hypothesis of NO being a senescence signal (Cam et al., 2012). In agreement with these data, Navascues et al. (2012) recently demonstrated that green Lb of soybean, characteristic of senescent nodules, is produced by the nitration of heme. Hence, these results provide evidence that reactive nitrogen species are produced during aging of legume nodules and further suggest that NO could also stimulate the senescence of determinate-type nodules. The way NO is involved in the senescence process remains the vast issue to be investigated. A transcriptional analysis revealed that more than 500 genes are regulated in senescent nodules of *M. truncatula* (Van de Velde et al., 2006), but it is not known yet those which are responding to NO. More recently, genetic experiments have allowed the identification of two new players in *M. truncatula* nodule senescence: a

transcription factor (MtNAC 969), which may negatively regulate nodule senescence, and a STAY-GREEN gene (MtSGR), whose expression is higher in senescent (natural or nitrate-induced) nodules as compared to young ones (de Zelicourt et al., 2012; Zhou et al., 2011). Nevertheless, the functional characterization of the genes acting in nodule senescence is very limited, and it remains to be determined whether these genes are also induced in the presence of NO.

64.3 NO LEVEL IN LEGUME NODULES: A MATTER OF BALANCE

NO can have a signaling or a toxic effect on cells depending upon its concentration. On the one hand, it can alter the expression of specific genes, therefore playing a key role in metabolism shift, defense, hormone signaling, development, and nodule senescence. On the other hand, it can inhibit enzyme activity and contribute to deleterious events and premature nodule senescence (see Figs. 64.1d, e and 64.3). In addition, NO is also present in soil, and if we suppose that the molecule is diffusing freely in plant roots, the concentration inside nodules must be rigorously controlled not only to avoid toxic effects but also to allow the signaling function(s) to occur.

In plants, Hbs are an important family of proteins known to fulfill the role of NO storage or scavenger (Gupta et al., 2011). Three major families of Hbs have been described in plants: Lbs, ns-Hbs, and truncated hemoglobins (tr-Hbs) (Gupta et al., 2011; Perazzolli et al., 2006; see Chapter 70). All plants examined so far contain one or more families of hemoglobins, which sometimes coexist, suggesting putatively different functions. Lbs are abundant (up to mM range) only in legumes and in a few other plants interacting with nitrogen-fixing bacteria (Hoy and Hargrove, 2008). Based on their affinity for oxygen and sequence homology, they belong to class 2 globins (Gupta et al., 2011). They are supposed to buffer free oxygen and have a key role in facilitating oxygen diffusion to N_2-fixing bacteria in root nodules to permit their active respiration without inhibition of nitrogenase activity. Besides this important function, deoxy-Lb has been shown to bind NO with a high affinity to form stable complexes in soybean (Herold and Puppo, 2005), and it has been proposed that Lb could act as a NO scavenger (Shimoda et al., 2009). This may also be a function of the ns-Hbs which occur in much lower concentration than Lb but are ubiquitous in the plant kingdom including symbiotic plants. Ns-Hbs are classified as belonging either to class 1 (ns-Hb1) or class 2 (ns-Hb2) based on their affinity for oxygen and sequence similarity (Hoy and Hargrove, 2008). Interestingly, ns-Hb1 could be part of a NO dioxygenase system, using traces of oxygen to convert NO to nitrate. NO has been shown to upregulate Hb expression in a number of plant species. For example, in the actinorhizal symbiosis between *Alnus firma* and *Frankia*, ns-Hb was strongly induced by the application of NO donors, and it was shown that *AfHb1*, as a NO scavenger, may support the nitrogen fixation ability of members of the genius *Frankia* (Sasakura et al., 2006). Treatment of *L. japonicus* with either *M. loti* or a purified lipopolysaccharide fraction from this bacterium or *M. sativa* with *S. meliloti* also transiently induces *ns-Hb1* expression and NO synthesis (Murakami et al., 2011; Nagata et al., 2008). On the other hand, it has been shown that nonsymbiotic rhizobia do not induce NO production and expression of *ns-Hb* genes (Nagata et al., 2008). The same group has shown that overexpression of *ns-Hb1* enhances symbiotic nitrogen fixation, suggesting that this occurs by removal of NO as an inhibitor of nitrogenase (Shimoda et al., 2009). Tr-Hbs have been shown to be induced in *Datisca glomerata* by inoculation with *Frankia* or in root nodules of *M. truncatula* elicited by *S. meliloti* (Pawlowski et al., 2007; Vieweg et al., 2005), leading the authors to propose a role for tr-Hbs in NO detoxification. Hence, plant hemoglobins are diverse, and it is emerging now that all hemoglobins may have a role to play, possibly at different stages of the symbiosis. We may have expected these molecules to be sufficient to control NO level, but recent results ruled out this hypothesis. Astonishingly, a bacterial strain of *S. meliloti* mutated in its flavohemoglobin gene (*hmp*) elicited nodules on *M. truncatula* roots which display higher levels of NO, a lower nitrogen fixation efficiency, and earlier nodule senescence than the WT (Cam et al., 2012). This strongly suggests that the expression of the bacterial hemoprotein is essential for maintaining NO levels compatible with symbiosis even though plant Hbs are proficient. Whether this bacterial hemoprotein is essential to control NO levels inside the bacteria or whether this hemoprotein is more efficient than plant Hb to degrade NO in nodules has to be investigated. A BLAST search of the *B. japonicum* genome using Hmp from *S. meliloti* revealed a gene (bll3766) which encodes a protein with only 35% identity with the one of *S. meliloti*. The search also revealed the presence of a single domain globin (sd-Hb) (blr2807) which has a high identity with sd-Hbs from *Vitreoscilla stercoraria* and *Campylobacter jejuni* implicated in NO detoxification. In *B. japonicum*, this globin was also shown to have a NO detoxification role under free-living, microaerobic conditions (Sanchez et al., 2011).

Hemoproteins are not the unique proteins involved in NO degradation in rhizobia. Indeed, in denitrifying bacteria, one candidate for NO degradation is the respiratory NO reductase (Nor) which catalyzes reduction of NO into nitrous oxide (N_2O). The best known is the cNor encoded by the *norCBQD* genes, which receive electrons from either c-type cytochrome or small blue copper proteins (azurin or pseudoazurin) (Hino et al., 2012). Under free-living denitrifying conditions, *B. japonicum* cNor is important for degradation of NO. However, inoculation of nitrate-treated soybean

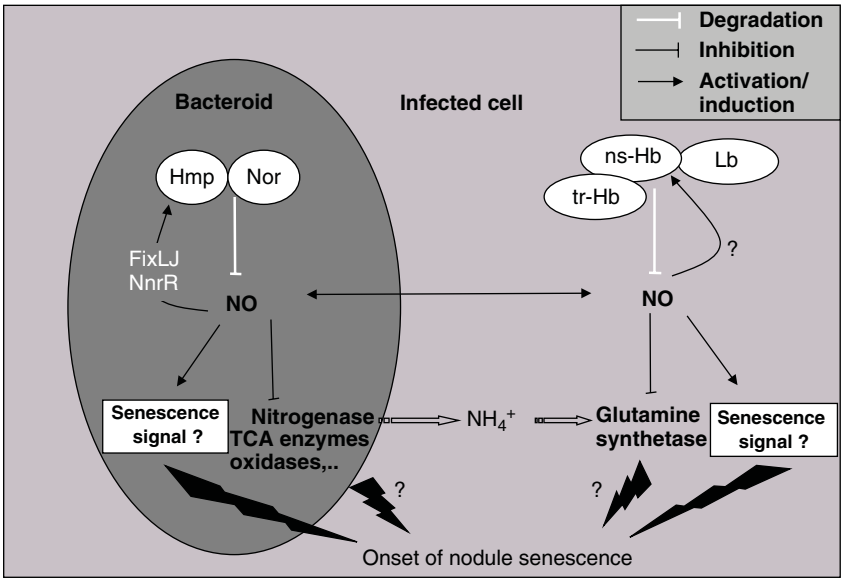

Figure 64.3 Schematic representation of known or putative molecular effects of NO leading to nodule senescence. Lb, leghemoglobin; ns-Hb, nonsymbiotic hemoglobin; tr-Hb, truncated hemoglobin; TCA, tricarboxylic acid cycle, Hmp, flavohemoglobin; Nor, NO reductase.

plants with a *norC* mutant does not affect the level of NO except in flooding stress conditions (Sanchez et al., 2010). *R. etli* lacks part of the denitrification pathway, and the presence of *norC* in this bacterium suggests a NO-degrading role for the enzyme. Indeed, Gomez-Hernandez et al. (2011) have shown that NorC is required to detoxify NO under free-living conditions; NO levels in nodules of common bean exposed to nitrate increased when elicited by a *norC* mutant as compared to the WT, indicating that Nor could also play a role in degrading NO in symbiosis. In *S. meliloti,* we have already shown that *nor* expression is induced in the presence of NO (Meilhoc et al., 2010). Preliminary results indicate that in free-living conditions, a *nor* mutant is more sensitive than a WT strain to a NO donor and triggers early senescence of *M. truncatula* nodules (Meilhoc et al., unpublished). It is interesting to note that Hmp does not compensate for the absence of Nor and vice versa, indicating that both degrading enzymes have either a specific function or a different nodule localization and both are needed to maintain an equilibrium in NO level. On the whole, both plant and bacterial proteins participate in maintaining NO balance at the different stages of symbiosis, and although the role of plant hemoglobins has been underlined for years, we should now consider bacterial NO-degrading enzymes as major components of this process (see Fig. 64.3).

64.4 CONCLUDING REMARKS AND FUTURE PROSPECTS

Even though NO has been known for decades to play major roles in mammals, it is only recently that NO was found to be also a pivotal molecule in plants. The presence of NO has been evidenced less than a decade ago in legumes–rhizobia interactions, and since then, studies on different legume models especially *Medicago*, *Lotus*, and soybean have established various roles of NO in these symbiotic interactions. How NO is generated is becoming better characterized; nevertheless, studies on the role of NO in legume nodules are still relatively limited by the lack of plant mutants with altered NO synthesis and the absence of noninvasive methods to assess *in vivo* NO status (Mur et al., 2011). Hence, experimental strategies to modulate NO levels on legume root or nodules rely on the use of NO donors or scavengers and of plant or bacterial mutants affected in NO-degrading proteins. NO donor/scavenger application may not be relevant regarding NO physiological concentration, but also their effects could be dependent upon the application method or even the type of NO donor/scavenger used (Filippou et al., 2012). Obtaining plant mutants affected in NO synthesis is a challenging perspective to avoid the use of NO donors.

The data summarized in this review indicate that NO has dual effects during symbiosis since it can have beneficial but also detrimental effects (e.g., on nitrogenase) (see Fig. 64.1). Indeed, the presence of NO is necessary for optimal infection/nodulation. Thus, NO could represent a specificity determinant for symbiont selection. Besides, it could also play a major role in repressing plant defense reactions in symbiotic conditions, thus favoring establishment of the beneficial plant–microbe interaction. In the same way, NO could also have a role to protect bacteria in the infection threads against the presence of hydrogen peroxide and/or trigger the expression of bacterial genes dedicated to adaptation to the higher NO level that bacteria could have to face at later stages of the symbiosis. In addition, we have observed that a bacterial strain which overexpresses a NO-degrading flavohemoglobin gene displays abortive infections threads (Cam et al., unpublished) which evokes

the hypothesis that NO could also be involved in infection thread development and stability. Finally, NO could also play a role in root cortical cell division and dedifferentiation and nodule formation (see Fig. 64.1c). Interestingly, a parallel can be made with the well-studied symbiosis between the squid *Euprymna scolopes* and the bacterium *Vibrio fischeri*, where NO has been shown to be a specificity determinant for successful colonization of the squid by the microsymbiont (Wang et al., 2010). Also in this interaction, NO acts as a signal molecule that prepares the symbiont to better survive oxidative and NO stresses by inducing the expression of a NO-degrading enzyme (Hmp) and a NO-resistant alternative oxidase (Davidson et al., 2004; Spiro, 2010; Wang et al., 2010; Wang and Ruby, 2010).

At later stages of the symbiosis (i.e., in mature nodules), NO has also been found to play a role in nodule energy metabolism and could act as a downregulator of N_2 fixation and carbon metabolism (by inhibiting nitrogenase and/or enzymes of the TCA cycle) to reduce energy demand in stress conditions such as a hypoxic environment. On the other hand, NO by inhibiting either nitrogenase or other essential plant metabolic enzymes (such as GS) or by inducing a yet unknown signal could promote early nodule senescence and interrupt symbiosis. Interestingly, in the coral forming symbiosis, the levels of NO found in host tissues strongly correlate with the extent of coral bleaching, leading to the hypothetical role of NO as a stress signal that mediates the breakdown of the symbiosis, in response, for example, to thermal stress (Bouchard and Yamasaki, 2008). More work remains to be done to better understand the plurality of NO effects and shed light on novel functions of this molecule at the different stages of symbiosis.

Moreover, most of the studies which have been done so far to elucidate the roles of NO at the different steps of the symbiotic interaction are mainly descriptive. The challenging issues in the coming years will be to uncover molecular targets of NO and decipher signaling pathways involving NO. Protein S-nitrosylation and tyrosine nitration are emerging as key redox-based posttranslational modifications in plants which are pivotal to convey NO bioactivity. For example, H. Carvalho's group carried out pioneering work showing that a GS of *M. truncatula* is inhibited by NO-mediated tyrosine nitration (Melo et al., 2011). In addition, a recent paper (Puppo et al., 2013) reports that *M. truncatula–S. meliloti* mature nodules contain at least 80 S-nitrosylated proteins, from either plant or bacterial origin, many of them being related to carbon and N_2-fixing metabolism. Some of these enzymes were also found to be inhibited by NO donors, that is, DEA-NONOate and S-nitrosoglutathione (GSNO) (aldolase, phosphoglycerate kinase, enolase, pyruvate kinase, phosphoenolpyruvate carboxylase, and asparagine synthetase; Brouquisse and Castella, unpublished). It may be noted that many of the proteins identified as being S-nitrosylated in the symbiotic interaction have also been reported to be S-sulfenylated in *M. truncatula* (Oger et al., 2012) or S-glutathionylated in *A. thaliana* (Dixon et al., 2005), suggesting that the same protein may be precisely and differentially regulated depending on redox state and signaling (Spadaro et al., 2010). Detailed studies of these proteins, the impact of NO on their activity, and their role in symbiosis should provide new insights in the signaling functions of NO.

Finally, as S-nitrosylated proteins are being identified as important intermediates in NO signaling, proteins involved in the regulation of these modifications will find all their importance since these modifications must be reversible to ensure transient signaling. GSNO reductase (GSNOR) and thioredoxins (Trx) are enzymes which could be involved in the reversal of S-nitrosylations (Malik et al., 2011; Sengupta and Holmgren, 2013). The *M. truncatula* genome contains a single GSNOR gene and 22 Trx genes among which two seem specific to legumes (Alkhalfioui et al., 2008). On the other hand, *S. meliloti* contains 3 GSNOR homologues, two thioredoxin genes (*trxA* and *trxC*) and one thioredoxin reductase gene (*trxB*), suggesting that both plant and bacterial systems could play a role in regulating protein S-nitrosylation. Studies on the role of these various proteins will permit to better understand how the NO signaling is transmitted and reversed.

The effect of NO should depend upon its spatiotemporal distribution/concentration in the nodule. Therefore, another challenging issue will be to assess precisely how much, where, and when NO is produced inside the nodule. Approaches based on the use of fluorescent probes have associated limits (Mur et al., 2011), and the use of NO-biosensor bacterial strains is restricted to bacteria-containing plant cells. Novel methods must be developed to accurately measure NO levels inside nodules and make it possible to better define the site(s) of NO generation. The development of experimental approaches designed to modify the NO level specifically in one of the different zones of the nodules (in the case of indeterminate nodules, see Fig. 64.1e) would create the possibility to shed light on the relevance of NO in each of these zones.

Undoubtedly, slight modifications in the NO level balance may impact the initiation or maintenance of the symbiotic interaction. Thus, lowering the NO level early in the process affects the infection and nodulation efficiency, while too much NO in mature nodules triggers their premature senescence. Therefore, the NO level should be tightly controlled at any time. Lbs as well as tr-Hbs and ns-Hbs may be committed to control NO level, but much remains to be done to clarify the role of each of these proteins at the successive steps of symbiosis. Besides, rhizobia possess NO-degrading enzymes such as flavohemoglobin and/or NO reductase, whose importance was not suspected until recently. These proteins cannot be overlooked as they emerge as essential to maintain NO level even though plant hemoglobins are

present. Other NO-degrading enzymes could be part of the bacterial NO response together with proteins important for bacterial adaptation to NO. Their role should be studied to better understand how bacteria contribute to maintain NO level and nitrogen fixation in nodules.

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Chapter 65

Profiling Symbiotic Responses of *Sinorhizobium fredii* Strain NGR234 with RNA-Seq

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65.1 INTRODUCTION

Proficient symbioses between legumes and soil bacteria known as rhizobia culminate with the development of root (or stem) nodules that provide host plants with reduced forms of atmospheric nitrogen (N_2). The series of events that leads to the formation of N_2-fixing nodules can be divided into two major consecutive processes: nodulation followed by nitrogen fixation. Nodulation covers the initial steps of the symbiosis and normally leads to the formation of nodules in which compatible rhizobia form persistent intracellular colonies. Transcriptional activation of rhizobial genes required for nodulation (*nod*, *nol* and *noe*) is mediated by specific regulators of the NodD family and cognate flavonoids that are released by host roots. Ultimately, nitrogen fixation occurs once rhizobia have successfully colonized the cytoplasm of nodule cells and differentiated into bacteroids that express the nitrogenase enzyme. Metabolism of endosymbiotic bacteroids differs considerably from that of free-living rhizobia, and these cellular adaptations can be monitored at the level of the proteome (pool of proteins) and/or transcriptome (total cellular RNA species), the latter of which may change rapidly in response to environmental cues.

Transcriptome profiling has become instrumental in understanding the molecular responses of isolated cells or tissues that are experimentally challenged. Until recently, transcriptome analyses were carried out using labeled-RNA or -cDNA samples (probes) hybridized against sets of DNA targets grouped in arrays that represented either selected parts or entire genomes. Initially prepared by lysing colonies or spotting PCR-amplified products on hybridization membranes, DNA-arrays were later miniaturized, and these so-called microarrays (also known as DNA chips, or biochips) soon became the reference tools in transcriptome analysis. In these hybridization systems, signal intensities (either fluorescent, or radioactive) correlated positively with gene expression since abundance of transcript species was maintained in the pool of DNA or RNA probes. Yet, microarrays suffered from a number of limitations including unspecific hybridizations (Wu et al., 2005: Zhang et al., 2005) contributing to false-positive signals (Chen et al.,

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2006), background noise and signal saturation that limited the dynamic range of detection (Dodd et al., 2004). With the development of massive parallel sequencing, also known as next-generation sequencing (NGS), it became possible (as well as affordable) to sequence pools of cellular RNAs and thus measure the number of copies of each RNA species. This method called RNA-Seq offers several advantages over microarrays. RNA-Seq was shown to quantify expression levels accurately, including for low abundance transcripts (Bashir et al., 2010; Mortazavi et al., 2008), and have – in principle – no upper quantification limit or saturation effect. Unlike microarrays that normally require annotated genome sequences for optimal design and construction, RNA-Seq was found appropriate for *de novo* transcriptomic or for completing transcripts inventories (Trapnell et al., 2010). Regardless of the NGS technology used (454 Sequencing, Illumina or SOLID), RNA molecules need generally to be converted into libraries of short cDNA fragments prior to sequencing. Thus, instead of few sequences of up to 1000 nucleotides long obtained with the "traditional" Sanger method, a single Illumina run may generate an output of tens of millions of sequence reads each of 50–150 bases in length. These short sequence-reads are then assembled *in silico* into longer transcripts, genes, and operons or alternatively mapped onto a genome, thus delimiting actively transcribed regions or by contrast, those that remained transcriptionally silent.

Transcriptomic studies in the field of rhizobia probably began with the analysis of the 536 kb long symbiotic plasmid (pNGR234a) of the broad host-range *Sinorhizobium fredii* strain NGR234. These analyses were carried out using DNA macroarrays of more than 400 PCR-amplified fragments and M13-phage subclones probed with radioactively labeled RNA isolated from free-living and endosymbiotic cells (Freiberg et al., 1997; Perret et al., 1999). In addition, macroarrays consisting of 921 M13 inserts of 1–1.5 kb collected to skim the genome of NGR234 (Viprey et al., 2000) were later probed in a similar manner (Perret et al., 2000b). Although relatively crude compared to today's data sets, these earlier results provided a solid basis for expression and functional studies of symbiotic open reading frames (ORFs) in NGR234 (Kobayashi et al., 2004; Perret et al., 2003; Viprey et al., 1998). In other pilot studies, the expression of up to 214 genes of *Sinorhizobium meliloti* strain 1021 was followed in as many as 10 experimental conditions using amplicons of circa 300 bp spotted by a robot onto nylon hybridization membranes (Ampe et al., 2003; Berges et al., 2003). Large DNA macroarrays consisting of circa 75,000 M13 subclones spotted on membranes were also used by Uchiumi and associates to identify a number of expression islands on the genome of *Mesorhizobium loti* strain MAFF303099 (Uchiumi et al., 2004). Microarrays of gene-specific amplicons (Becker et al., 2004) or oligonucleotide-based gene chips (Barnett et al., 2004; Domínguez-Ferreras et al., 2006) greatly facilitated the probing of entire rhizobial genomes, including for intergenic regions initially annotated as non-protein-coding. In fact, microarrays remained for many years the technology of choice for transcriptome analyses in various rhizobia, including for *Bradyrhizobium japonicum* strain USAD110 (Masloboeva et al., 2012; Pessi et al., 2007), *Rhizobium etli* strain CFN42 (Vercruysse et al., 2011), and *Rhizobium leguminosarum* biovar *viciae* strain 3841 (Karunakaran et al., 2009). In contrast, and except for a genome-wide survey of small untranslated RNAs (sRNAs) in *S. meliloti* (Schlüter et al., 2010), RNA-Seq was mainly used to analyze simultaneously the transcriptional behavior of nodule cells and bacteroids in associations between soybean and *B. japonicum* (Severin et al., 2010) or *Medicago truncatula* and *S. meliloti* (Boscari et al., 2013).

The competitive costs of NGS technologies and a recently completed genome sequence for strain NGR234 (Schmeisser et al., 2009), prompted us to use RNA-Seq to extend to the 3.9 Mb chromosome and 2.4 Mb mega-plasmid pNGR234b the initial transcriptomic studies carried out on the smaller 536 kb pNGR234a. In order to compare RNA-Seq data with results from previous analyses, total RNA was isolated from free-living cells of *S. fredii* strain NGR234 grown *in vitro* for 6 h in the absence (condition 1) or presence (condition 2) of the flavonoid daidzein, and (3) from nitrogen-fixing nodules collected on roots of the host plant *Vigna unguiculata* 38 days post-inoculation (dpi) with NGR234. Experimental conditions 1 and 2 were designed to examine the response(s) of NGR234 to the presence of a flavonoid known to induce the expression of nodulation genes, while condition 3 provided a measure of the transcriptional activity inside N_2-fixing bacteroids of NGR234. The following sections describe the experimental setups, bioinformatic and validation analyses that were carried out on the resulting RNA-Seq datasets, and some of the findings that illustrate how NGR234 responds to symbiotic cues from host plants.

65.2 METHODS

65.2.1 Growth Conditions

Cells of *S. fredii* NGR234 were grown at 27 °C in *Rhizobium* minimal medium supplemented with 12 mM succinate (RMS) and rifampicin at 50 µg/ml (Broughton et al., 1986). For flavonoid induction, cells from fresh overnight pre-cultures were diluted to an OD_{600} 0.1 prior to the addition of daidzein at a final concentration 2×10^{-7} M. As daidzein was prepared as a stock solution of 10^{-3} M diluted in ethanol, a volume of ethanol equivalent to that of diluted daidzein was added to non-induced cultures to minimize any experimental bias. Following 6 h of growth

in the presence or absence of daidzein, free-living cells (at $OD_{600} \approx 0.3$) were harvested by centrifugation at 4 °C prior to RNA isolation.

65.2.2 Plant Assays

Seeds of *V. unguiculata* (L.) Walp. were surface sterilized and germinated for 3 days in the dark at 27 °C. Seedlings were then planted in Magenta jars containing vermiculite and nitrogen-free B&D as nutrient solution (Lewin et al., 1990). Following an inoculation with 2×10^8 bacteria, plants were grown for 38 days at a day temperature of 27 °C, a night temperature of 20 °C, and a photoperiod of 12 h with maximum light intensity of 100 µE/m^2/s.

65.2.3 Isolation of Nodule Bacteria

Once collected on roots of mature plants, nitrogen-fixing nodules was rapidly processed as follows: 1 g of fresh nodules were ground in 20 ml ice-cold MES buffer (350 mM Mannitol, 25 mM 2-[N-morpholino]ethanesulfonic acid, 3 mM $MgSO_4$ $7H_2O$, pH 7) (Natera et al., 2000). To remove most plant debris, the homogenate was filtered through four layers of moist Miracloth and centrifuged at 1000 g for 3 min at 4 °C. The supernatant was transferred into a new tube and centrifuged at 8000 g for 12 min at 4 °C. The crude cell pellet containing bacteroids was resuspended in the lysis phenol-NETS buffer for RNA preparation.

65.2.4 RNA Preparation and High-Throughput Sequencing

Total RNA was extracted from 5×10^9 to 5×10^{10} cells of NGR234 using a 5 ml mixture of equal volumes of pH 4.3 phenol (Sigma-Aldrich, St-Louis, Missouri, USA) and NETS 2X buffer (NaCl 400 mM, Tris-HCl 20 mM pH 8.0, EDTA 1 mM, SDS 1% w/v) that was prewarmed in a boiling waterbath. The mixture was vortexed for 1 min, incubated for 30 s in a boiling waterbath, and then centrifuged at 16,000 g for 5 min at 4 °C. Following a second extraction with an equal volume of hot phenol at pH 4.3, the aqueous phase was then extracted with an equal volume of phenol/chloroform/isoamyl alcohol 50:49:1 (vol/vol) (Sigma-Aldrich, St-Louis, Missouri, USA), and ultimately precipitated using 1/3 vol of 8 M LiCl and 2 vol of ethanol. Following centrifugation, the enriched-RNA pellet was resuspended in 500 µl DEPC-treated and RNAse-free water, extracted twice with phenol/chloroform/isoamyl alcohol 50:49:1 (vol/vol), and precipitated using 1/10 vol Na-acetate 3 M, pH 5.2, and 2 vol ethanol. After centrifugation, the RNA pellet was washed with 70% ethanol, dried, and resuspended in 110 µl DEPC-treated and RNAse-free water. Contaminating genomic DNA was removed with two consecutive DNase I treatments using the RNeasy mini protocol (QIAGEN GmbH, Germany). Total RNA samples were examined on agarose gel for possible degradation, and their concentration and purity were determined by spectrophotometry. For depleting RNA preparations of 16S and 23S ribosomal RNA (rRNA), samples of maximum 7 µg were treated twice with the MicrobExpress™ bacterial mRNA purification kit (Ambion, Applied Biosystems, Carlsbad, California, USA). The effect of rRNA depletion on sample quality was verified using qRT-PCR. For normalization purposes, each of the RNA samples analyzed by high-throughput sequencing was a mixture of three independent RNA preparations and biological replicates. Enriched mRNA samples were given to Fasteris SA (Geneva, Switzerland) for cDNA library construction and sequencing with an Illumina HiSeq-2000 platform that ultimately yielded 50-mer paired-end sequencing reads. The major characteristics of the resulting libraries of sequence reads are listed in Table 65.1.

65.2.5 Mapping of Sequence Reads and Statistical Analysis

Reads were mapped onto the three replicons of the NGR234 genome [symbiotic plasmid pNGR234a (NC_000914.2), megaplasmid pNGR234b (NC_012586.1), and chromosome (NC_012587.1)] using BWA 0.5.8 software (Li et al., 2008) and allowing for a maximum of two mismatches in the 32 bp seed ($-l$ and $-k$ options respectively) and four mismatches on the total read sequence of 50 bases ($-n$ option). To reduce complexity, sequence reads mapping into the 5S (NGR_c26480, _c30320, c34260), 16S (NGR_c26520, _c30360, c34210), and 23S (NGR_26490, _c30330, c34240) rRNA genes were discarded from the analyzed dataset. Normalization of read counts and quantification of gene expression were performed using Seqmonk 0.22.0 (http://www.bioinformatics.babraham.ac.uk/projects/seqmonk/). To normalize datasets, each of the three libraries was scaled up to the same level, and read counts were normalized according to the length of targeted genes or transcripts. Relative expression levels were calculated from the resulting output files using scripts written in the statistical computing environment R (R Development Core Team 2013).

65.2.6 Real-Time Quantitative Reverse Transcription (qRT-PCR)

cDNA amplifications and qRT-PCR experiments were carried out as described previously (Fumeaux et al., 2011). Briefly, primers for amplicons of 100–150 bp were designed using the online Primer3 software (Rozen and Skaletsky, 2000) and are listed in Table 65.2. Reverse transcription was performed in a final volume of 20 µl with 1 µg of DNase-treated RNA, 1 µM of random hexamers, and the "iScript™ Select cDNA Synthesis Kit" (BioRad), during

Table 65.1 Major characteristics of Illumina datasets

Experimental Conditions		RMS	RMS + Daidzein	Nodules
Number of sequence reads		44,442,726	41,429,214	50,862,542
Non-NGR234 sequence reads (% of total)		2.30	1.21	88.94
Reads of 16S, 23S, and 5S rRNA (% of total)		86.42	84.20	9.99
Reads mapping elsewhere on the NGR234 genome	% of total reads	11.28	14.59	1.07
	Number of reads	5,011,460	6,045,772	543,803
	Mb equivalent	250.6	302.3	27.2
	Fold coverage	36.4	43.9	3.9

30 min at 42 °C. qRT-PCR amplifications were performed in duplicate in 20 µl of reaction mixes containing 5 µl of 10× diluted cDNA template, 10 µl of SYBR Green Supermix (Bio-Rad), and 500 nM of each primer. After an initial activation step of 3 min at 95 °C, the Biorad iCycler was programmed for 40 cycles of 30 s at 95 °C and 1 min at 60 °C, with ultimately a melting-curve analysis of PCR products to verify the specificity of amplifications. Values of relative gene expression are the means of three independent RNA preparations with one qRT-PCR for each RNA preparation, and were normalized against 16S rRNA.

65.3 RESULTS AND DISCUSSION

Major characteristics of the RNA-Seq datasets are summarized in Table 65.1. In spite of two consecutive ribosomal RNA depletion steps prior to cDNA synthesis, sequence reads corresponding to 5S, 16S, and 23S rRNA were six to nine times more abundant than non-rRNA transcripts. Mao and associates reported a similar observation when carrying out a pyrosequencing survey of the transcriptome of *S. meliloti* strain 1021 cells grown to mid-exponential phase ($O.D._{600} \cong 0.6$) (Mao et al., 2008). To reduce complexity and sizes of the datasets, sequence reads mapping into 5S (NGR_c26480, _c30320, c34260), 16S (NGR_c26520, _c30360, c34210), and 23S (NGR_26490, _c30330, c34240) rRNA genes were initially discarded. However, to preserve some information on transcription of the three rRNA operons, reads mapping into the shorter 16S–23S and 23S–5S intergenic regions, as well as upstream of the 16S and downstream of the 5S loci were not removed from datasets. As shown in Table 65.1, non-ribosomal RNA species from free-living cells of NGR234 grown in the presence or absence of daidzein (RMS and RMS + daidzein) still represented a total of 250–300 Mb of sequence data that was equivalent to a 36- to 44-fold genome coverage. In order to minimize the impact of the isolation procedure on the transcriptome of bacteroids inside 38 dpi nodules of *V. unguiculata*, the total RNA was rapidly isolated from crude cell pellets. As a consequence more than 80% of the Illumina reads collected from the nodule samples were mostly of

Table 65.2 List of target genes and primers used for qRT-PCR

Targeted Gene	Primer Name	Primer Sequence (5′ to 3′)	Amplicon (bp)
NGR_b06210	RT1-NGR_b06210	CGCATATCACCAACAAGCTG	120
	RT2-NGR_b06210	ACCGCCTGTGTCAGTTCTTC	
nifH	RT1-nifH	CAATTCCAGGCTCATCCACT	142
	RT2-nifH	GGAATTGGCATGGATCTTGT	
nifS	RT1-nifS	GATGCTGCCATTCTTTGCAG	145
	RT2-nifS	GTGATCTCTGGGTCGAACTT	
groEL1	RT1-groEL1	GCTTCGAAGACCAACGACAT	138
	RT2-groEL1	CTTCAGGTCCATCGGGTTC	
rplL	RT1-rplL	CGCAAAGATCGTTGAAGACC	181
	RT2-rplL	GATGACGTCGAACTCGGTCT	
rpsA	RT1-rpsA	GATTTCGCAGCACTTCTCGA	151
	RT2-rpsA	CGAATTCCTTGAGCGGAACG	
sigA (rpoD)	RT1-sigA	ACATCATCGCCGAGACCTAC	135
	RT2-sigA	CCTTGATCAGCTCGTCCTTC	
sucD	RT1-sucD	CACCAAGGTCCTCGTTCAG	157
	RT2-sucD	GAAGATCGGCAGGGTTTCG	

plant origin (data not shown) and are not discussed here. Once non-NGR234 sequence reads or those matching rRNA species were removed, the bacteroid dataset still included more than half a million reads, which together covered >27 Mb of sequence data or four times the NGR234 genome size. Although the pools of non-rRNA reads from free-living bacteria or bacteroids differed significantly in size (see Table 65.1), qRT-PCR validation experiments described further below confirmed that bacteroid data was sufficient to provide reliable expression values. For subsequent calculations of gene expression, the three libraries of sequence reads were scaled up to the same level, and read count was adjusted for gene length.

65.3.1 Core Versus Symbiotic Genomes

Once sequence reads were mapped onto each of the three replicons of the NGR234 genome, subsequent analyses were carried out using SeqMonk, a free software developed by Babraham Bioinformatics for the visualization and analysis of large mapped datasets. For each of the three experimental conditions tested, the respective contributions of the NGR234 replicons to the pool of non-rRNA transcripts were calculated. As shown in Figure 65.1, more than 85% of the sequence reads mapped onto the chromosome of 3.9 Mb when cells were actively dividing in free-living conditions. This result was consistent with the NGR234 chromosome carrying most elements of the "core genome" as was initially described for *R. leguminosarum* biovar *viciae* strain 3841 (Young et al., 2006). In contrast, the respective contributions to free-living transcripts of the symbiotic plasmid pNGR234a (1.5%) and mega-plasmid pNGR234b (10.9%) to the total number of sequence reads were minor, as befit "accessory components." Yet, the addition of daidzein to the growth medium increased significantly the contribution of pNGR234a to 3.3% of total sequence reads because of the flavonoid- and NodD1-dependent activation of symbiotic genes (Kobayashi et al., 2004; Perret et al., 1999). Contribution of pNGR234a to the pool of transcripts increased even more dramatically (up to 35%) when bacteroids fixed nitrogen inside plants, illustrating further the clear symbiotic role of the smallest of the three replicons. In contrast, the contribution of the chromosome to the bacteroid lifestyle was considerably reduced (down to 51% of total reads), while that of pNGR234b remained almost constant. Initially observed in NGR234 (Perret et al., 2000b) and *M. loti* strain MAFF303099 (Uchiumi et al., 2004), downregulation of many chromosomal and house-keeping functions inside N_2-fixing bacteroids was later proposed to result mainly from growth arrest (Capela et al., 2006; Vercruysse et al., 2011). Apparently, this phenomenon was found to be common to bacteroids of nodules of determinate (bean, cowpea and *Lotus*) or indeterminate (alfalfa, and pea) types, and also occurred in endoreduplicated cells of *S. meliloti* and *R. leguminosarum* (Capela et al., 2006; Karunakaran et al., 2009).

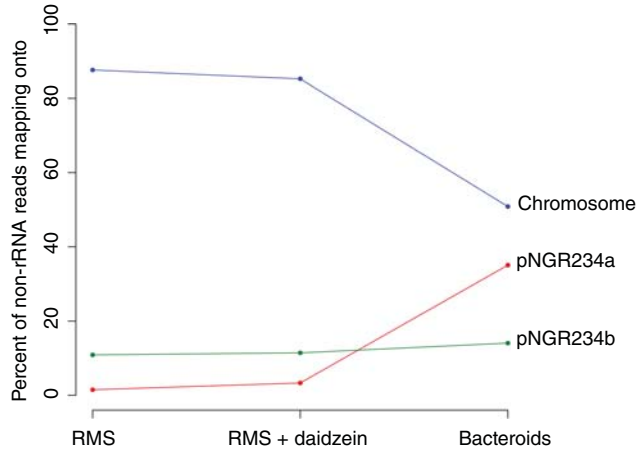

Figure 65.1 Respective contributions of the NGR234 replicons to non-rRNA transcriptome. Sequence reads from RNA-Seq datasets were mapped onto the NGR234 genome using BWA 0.5.8 software. The proportion of total reads mapping on the chromosome (blue line), symbiotic plasmid pNGR234a (red line), or mega-plasmid pNGR234b (green line) are given for each of the three experimental conditions tested: free-living growth in RMS (RMS), or RMS supplemented with 2×10^{-7} M daidzein (RMS + daidzein), or the endosymbiotic lifestyle (Bacteroids).

65.3.2 Validation of RNA-Seq Data

For each experimental condition and to normalize sample preparations, a mixture of three independent RNA preparations and biological replicates were pooled prior to the synthesis of cDNA libraries. Ultimately, to confirm that no significant bias was introduced during the construction of cDNA and sequencing libraries for RNA-Seq, the expression with more than 25 reference loci was followed by qRT-PCR. This validation step seemed particularly important for the RNA-Seq exploration of the bacteroid transcriptome, since the corresponding dataset was reduced as compared to conditions 1 and 2 (see Table 65.1). Several criteria were considered for selecting the most appropriate reference genes, mainly: (i) the functions of the encoded proteins, (ii) the relative expression levels in the three experimental conditions, and (iii) whether these loci were chromosomal or plasmid born. Although results were conclusive for all of the selected targets, Figure 65.2 shows the Log2 fold-change expression values measured for a subset of eight reference genes when cells of NGR234 were either growing in RMS (condition 1) or fixing nitrogen in association with *V. unguiculata* (condition 3). These eight selected genes code for the major 60 kDa chaperonin GroEL1 (NGR_c04080), ribosomal proteins RplL

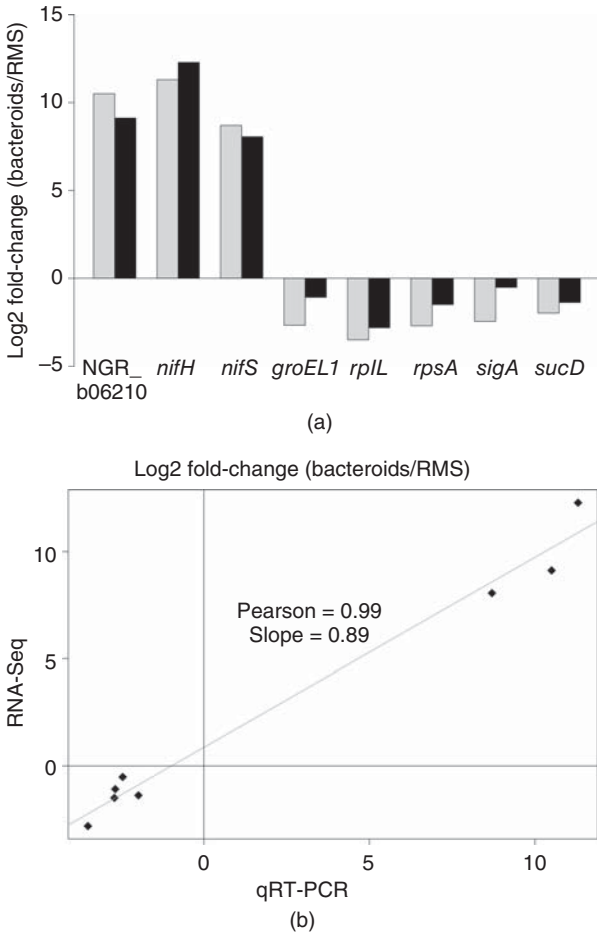

Figure 65.2 Changes in gene expression monitored with RNA-Seq and qRT-PCR. Differential expression of eight reference genes in free-living cells and bacteroids of NGR234 was compared using RNA-Seq and qRT-PCR data. Cells of NGR234 were grown in minimal medium supplemented with succinate to an $OD_{600} \cong 0.6$ (RMS) or isolated from 38 days-post-inoculation (dpi) nodules of *V. unguiculata* (Bacteroids). Changes in gene expression were calculated as the log2 fold-change using RMS as the reference condition. Expression values were the means of at least three independent qRT-PCRs, each using a different RNA preparation as template. Panel (a) shows for each of the eight reference loci the log2 fold-change in gene expression calculated using either normalized RNA-Seq datasets (black bars) or qRT-PCR results (gray bars). Panel (b) shows the result of a Pearson's R correlation test carried out on the log2 fold-change data calculated for each reference gene using either the RNA-Seq or qRT-PCR data. The resulting Pearson R value is 0.99 with a distribution of data points close to linear and a resulting slope of 0.89.

(NGR_c11820) and RpsA (NGR_c35860), sigma-70 (SigA or RpoD) factor (NGR_c22670), succinyl-CoA synthetase SucD (NGR_c31280), an oxidoreductase (NGR_b06210), as well as the nitrogenase iron protein NifH (NGR_a00890 and _a01130) and cysteine desulfurase NifS (NGR_a00930). Together, these genes belong to various functional and phenotypical classes, as well as distinct expression profiles. While expression of *nifH* and *nifS* was clearly restricted to bacteroids, transcription of many housekeeping loci during symbiotic nitrogen fixation was clearly downregulated. Although values calculated with RNA-Seq or qRT-PCR data were not necessarily identical, they were strongly correlated with a Pearson coefficient close to 1 (Fig. 65.2b). These results confirmed the quality and reliability of the RNA-Seq data collected for the three experimental conditions we chose to investigate.

65.3.3 Flavonoid-Dependent Modulation of Gene Expression

As discussed previously, and extensively reviewed elsewhere (Gibson et al., 2008; Perret et al., 2000a), flavonoids released by plant roots play an important signaling role in the molecular dialogue host plants establish with rhizobia (see Chapter 50). Flavonoid-dependent activation of rhizobial nodulation genes is mediated by transcriptional regulators of the NodD family that bind to conserved regulatory elements of 49 bp called *nod*-boxes (NB) found upstream of *nod* genes or operons. With 19 functional NB (NB1 to NB19) dispersed over pNGR234a (Freiberg et al., 1997;

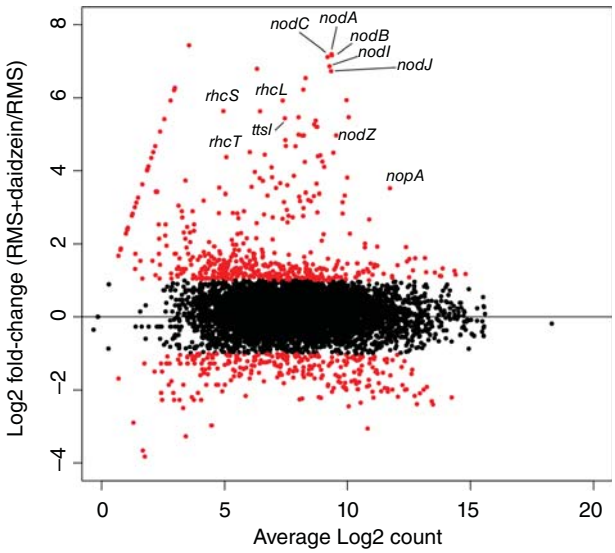

Figure 65.3 Global response of NGR234 cells to the presence of inducing-daidzein in the growth medium. For each of the annotated genes of strain NGR234, the normalized expression values measured in free-living bacteria growing in minimal medium in the absence (RMS) or presence of daidzein (RMS + daidzein) were averaged and transformed into a $\log_2$ count (average Log2 count), and plotted against $\log_2$ transformed fold-change values measured for the same experimental condition [Log2 fold-change (RMS + daidzein/RMS)]. The 754 genes displaying a |fold-change value| ≥ 2 are shown as red dots, while those whose expression varies less than the selected threshold are marked as black dots. Respective positions of a number of important nodulation (e.g., *nodABCIJZ*) and T3SS-related (*nopA*, *rhcLST*, and *ttsI*) genes are also shown.

Kobayashi et al., 2004; Perret et al., 1999), strain NGR234 has more functional NB than any other fully sequenced rhizobial strain. Detailed functional analysis showed that a NodD1-TtsI-SyrM2-NodD2 regulatory cascade controls the sequential activation of at least 75 genes in NGR234, many of which are found downstream of NB1 to NB19. Other flavonoid-responsive loci, such as those coding for a symbiotically active type three secretion system (T3SS) (Viprey et al., 1998), are regulated by TtsI-binding sequences called *tts*-boxes (TB1 to TB11) (Marie et al., 2004). In fact, the capacity of NodD1 to be activated by a broad panel of inducing flavonoids (Le Strange et al., 1990) was proposed to be one of the major keys that allowed NGR234 to initiate a symbiotic dialogue with a broad spectrum of plants (Perret et al., 2003; Perret et al., 2000a; Pueppke and Broughton, 1999). To examine how NGR234 responded to the presence of NodD1-compatible flavonoids, 2×10^{-7} M daidzein was added to the growth medium (experimental condition 2, RMS + daidzein) and cells collected for RNA-Seq analysis after 6 h incubation at 28 °C. Although activity of the NGR234 flavonoid-dependent regulatory cascade was shown to extend over 48 h, most nodulation genes were actively transcribed at 6 h post-induction (Bakkou, 2011; Kobayashi et al., 2004). Control cells were grown in the same conditions (condition 1, RMS), except for the inducing daidzein-ethanol solution that was replaced by an equivalent volume of ethanol (10^{-4} vol. of growth medium). Figure 65.3 shows how daidzein affected transcription of many of the annotated genes of NGR234, with loci having a |fold-change value| ≥ 2 highlighted in red. As expected, the *nodA*, *nodB,* and *nodC* genes for which expression was shown to be maximal after 6 h of daidzein induction, were found in the upper part of the plot. In contrast, maximal transcription of genes coding for components of the T3SS (e.g., *rhcL*, *rhcS,* and *rhcT*) as well as those coding for nodulation outer proteins (Nop) was expected to be achieved later than 6 h post flavonoid induction. Interestingly, RNA-Seq data indicated that in addition to loci directly controlled by the NodD1-dependent cascade many more genes (754 in total with a cutoff |fold-change value| ≥ 2) responded positively or negatively to the presence of inducing flavonoids in the growth medium. Although this result suggested that a purified flavonoid had a broad impact on the metabolism of NGR234, it seemed unlikely that most of these flavonoid-responding loci played a significant role in symbiosis. Nonetheless, a number of chromosomal and pNGR234b gene clusters previously unknown are currently the targets of more detailed molecular analyses.

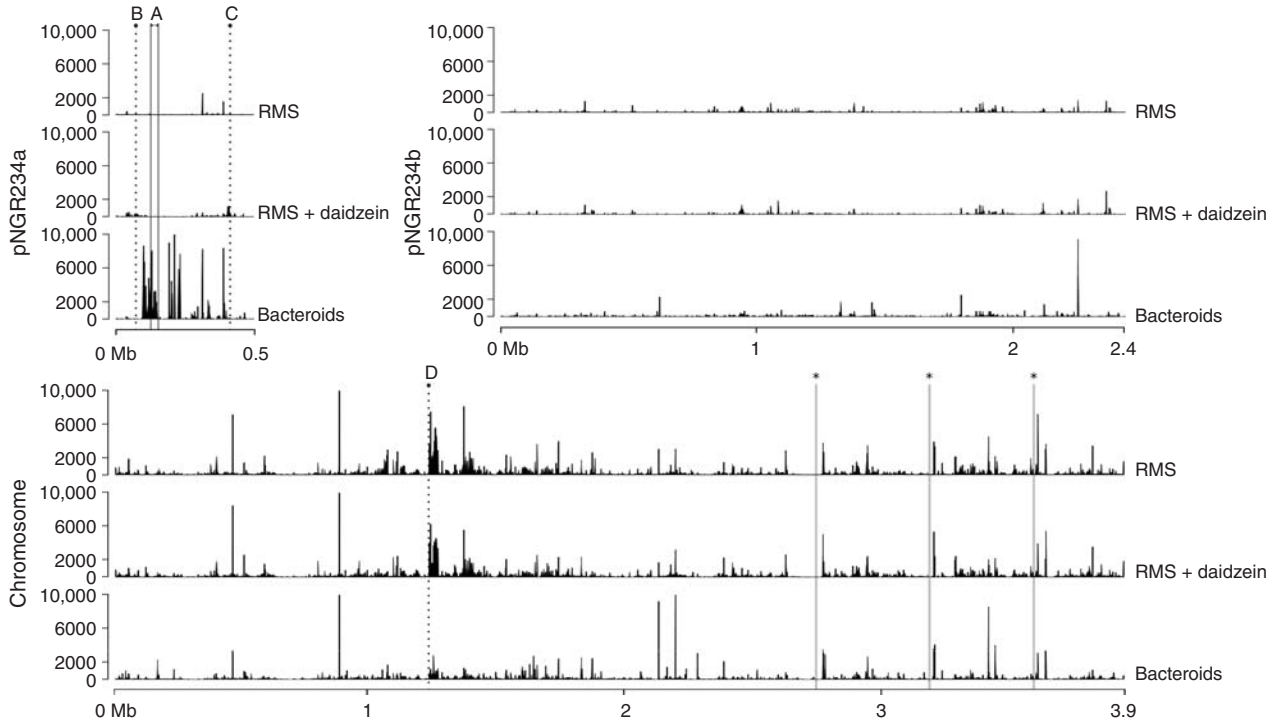

Figure 65.4 Transcription profiles of the three NGR234 replicons. To provide a measure of expression on a given section of a replicon, the number of read counts was averaged over contiguous windows of 1000 nucleotides in size and plotted on to the scale linear representations of each replicon: chromosome, symbiotic plasmid pNGR234a, or mega-plasmid pNGR234b. Read counts were normalized for the total number of sequence reads and the few values above 10,000 were not reported to ensure a better readability. Positions of the regions of particular interest (A, B, C, and D) shown at a higher resolution in Figure 65.5 are marked with dashed lines. Positions on the chromosome of the three rRNA operons are marked with asterisks.

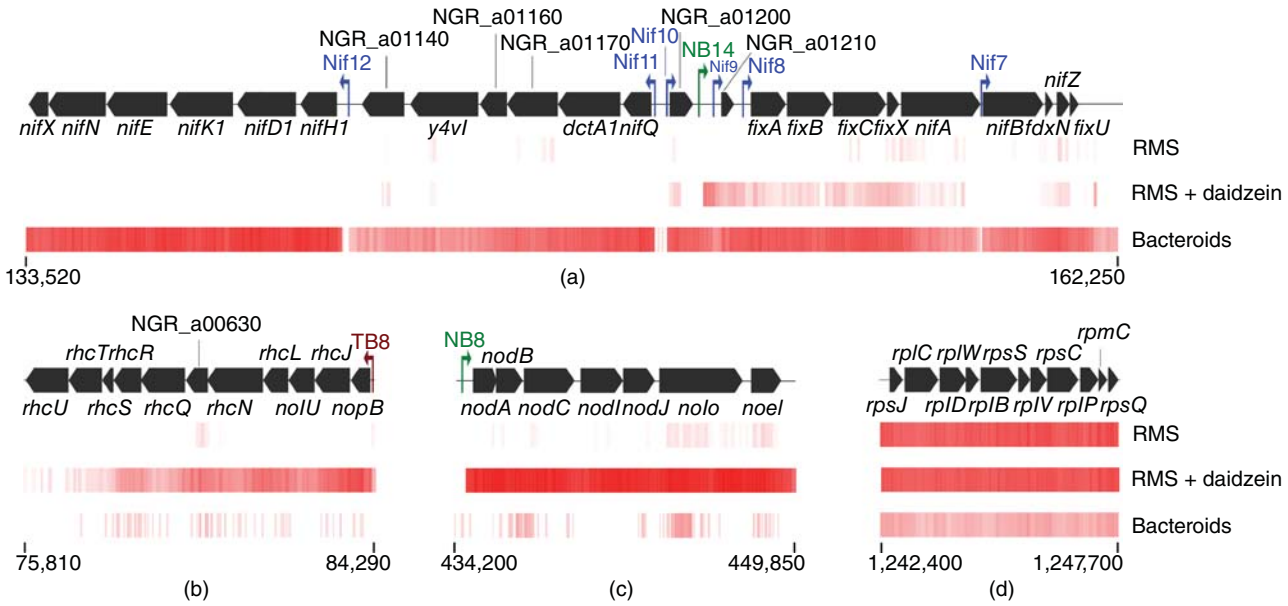

Figure 65.5 Correlated expression and genetic maps for the NGR234 genome. Three sections of pNGR234a [(a), (b) and (c)] and one of the chromosome (d) were selected to illustrate variations in gene expression in the three experimental conditions analyzed by RNA-Seq: free-living cells grown in minimal medium in the absence (RMS) or presence of daidzein (RMS + daidzein), or bacteroids found in 38 dpi nitrogen-fixing nodules of cowpea (Bacteroids). For each of the corresponding sections of the NGR234 genome, a genetic map shows the positions of annotated ORFs and known symbiotic promoters such as *nod*-boxes (NB), TtsI-binding boxes (TB), or NifA-σ^{54} promoters (Nif). These regulatory elements are numbered as in Perret et al. (2003). The intensity of gene expression was represented as a heat map using contiguous windows of 100 nucleotides, and 256 shades of red ranging from white (no expression) to dark red (averaged nucleotide coverage over 100 bases >16,865 or >41,235 for pNGR234a or the chromosome respectively). The respective positions of sections (a), (b), (c) and (d) on the symbiotic plasmid or chromosome were also highlighted in Figure 65.4.

65.3.4 Transcriptional Landscapes in NGR234

As discussed above, the replicon's contribution to the pool of cellular transcripts was considerably influenced by the environmental conditions encountered by bacteria. For example, the presence of daidzein in the growth medium that triggered the expression of nodulation genes, had little effect on the global transcriptional landscape of NGR234 (see Fig. 65.4, RMS and RMS + daidzein). In contrast, differentiation into N_2-fixing bacteroids resulted in an important shift of transcription from many chromosomal functions to regions of pNGR234a responsible for symbiotic nitrogen fixation (Fig. 65.4, RMS and Bacteroids). Interestingly, transcription profiles in free-living cells showed that strong gene expression was often restricted to narrow islands of the chromosome and did not necessarily coincide with the rRNA operons. At a higher resolution, RNA-Seq data also provided single-gene expression values. Instead of poorly informative spreadsheet tables that are difficult to read, transcription and genetic data were assembled into a single high resolution map that covered the three NGR234 replicons and for the three experimental conditions we analyzed (see Fig. 65.5). For this expression map that can be queried via a web-based platform, the normalized RNA-Seq data was converted into a series of contiguous expression windows of 100 nucleotides in size that were aligned with the annotated genome of NGR234. Transcription levels were converted into a colored heat map of 256 shades of red ranging from white (no expression) to dark red (maximal expression for a selected replicon). By scrolling over the transcription map in a particular region, the averaged expression value can be seen for each of the 100 bp-long windows. In addition, positions of known symbiotically active promoters such as the 19 NB, 11 *tts*-boxes, and 16 NifA-sigma 54 regulatory regions of pNGR234a were included. As seen in panels A, B, and C of Figure 65.5, flavonoid-dependent transcription correlated extremely well with the presence of *nod*- or *tts*-boxes, whereas bacteroid-specific transcription often coincided with NifA-σ^{54} types of promoters. In contrast, panel D illustrates the pseudo-constitutive expression of several genes coding for ribosomal proteins (*rpsJ* to *rpsQ*), all of which were downregulated once bacteroids fixed nitrogen. Values reported in Table 65.3 correspond to the normalized expression values calculated for four of the genes shown in Figure 65.5, and highlight the strict regulation of symbiotic genes such as *nodA*, *nopB,* or *nifE*, in contrast to *rplC* that was expressed in all conditions.

Table 65.3 Normalized gene-expression values for a few selected loci

Gene	Function	Normalized Expression Value in RMS	RMS + Daidzein	Bacteroids
nodA	*nod*-factor biosynthesis	9.1	1311.8	3.1
nopB	Constituant of T3SS pilus	5.6	422.4	14.9
nifE	Nitrogenase component	3.5	1.7	6365.2
rplC	50S ribosomal protein L3	2916.3	3091.2	380.1

For all of the annotated genes of NGR234, expression values for all three experimental conditions were normalized and calculated as in the Section 65.2.

65.3.5 Conclusions

In addition to single-gene expression values, RNA-Seq provides a wealth of information that cannot be easily obtained using transcriptomic tools such as conventional microarrays or DNA chips. Given that enough sequence reads are collected for transcribed regions to be extensively covered, transcription units, operon structures, transcription starts, and positions of cognate promoters can be determined. In fact, transcription units predicted for pNGR234a by earlier bioinformatic studies (Perret et al., 2003) matched well those observed in the global expression map obtained for NGR234, and for which Figure 65.5 illustrates a few telling examples. Except for its still elevated costs, which limit the number of replicates or experimental conditions one can afford to test, RNA-Seq suffers from a few drawbacks. One of these limitations is linked to the presence of numerous multicopy and nearly identical loci found in many rhizobial genomes. In fact, the NGR234 genome was shown to carry numerous repeated sequences, many of which are identical in sequence and code for transposable elements (Freiberg et al., 1997; Perret et al., 2001; Perret et al., 1997; Schmeisser et al., 2009). During the automatic mapping procedure, each sequence read that belonged to a repeat was randomly assigned to one of the multiple copies. In the case of the duplicated *nifHDK* genes that are controlled by two functional promoters of nearly identical sequences (Badenoch-Jones et al., 1989; Freiberg et al., 1997), this has no significant consequence on the true-expression value calculated for each gene copy. However, in the case of an insertion sequence such as NGRIS-13 (1573 bp long) that is found in nine identical copies on the chromosome, the random mapping of corresponding sequence reads significantly biased expression values for the gene within the IS element and for those found immediately upstream and downstream of the integration site. Most probably, transcription reported for the various NGRIS-13 copies resulted from a read-through of a single and strong promoter that was adjacent to only one of the nine transposable elements. This example shows that although RNA-Seq data provides a wealth of data, expression values must be analyzed with care and correlated with the genomic context into which transcription of a given gene or operon takes place.

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Chapter 66

Computational and Experimental Evidence That Auxin Accumulation in Nodule and Lateral Root Primordia Occurs by Different Mechanisms

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66.1 INTRODUCTION

The plant body plan is highly adaptive: new organs are created according to need. Legume roots form a particularly interesting example with respect to lateral organ initiation: they can form different types of organs, lateral roots, and root nodules. Although the latter are initiated in response to rhizobium-secreted signaling molecules, Nod factors, and the primordia of the two types originate in different cell layers, accumulation of the plant hormone auxin at the site of primodium initiation occurs in both (Rolfe et al., 1997; Larkin et al., 1996; Mathesius et al., 1998; Pacios-Bras et al., 2003; Takanashi et al., 2011). For a general review on the role of hormones in nodulation (see Chapter 56). Given the importance of auxin in the process (see Chapter 47), it is not surprising that the number of lateral roots can be increased by exogenous auxin application (Blakely and Evans, 1979; Woodward and Bartel, 2005). This is, however, not reported for root nodules. Auxin's textbook antagonist, cytokinin, on the other hand, can be used to induce nodule-like structures (Cooper and Long, 1994), as can auxin transport blockers (Hirsch et al., 1989). A cytokinin receptor is essential for nodulation (Gonzalez-Rizzo et al., 2006; Tirichine et al., 2007, Murray et al., 2007; Plet et al., 2011) and cytokinin response regulators are activated early in the process (Op den Camp et al., 2011). This further demonstrates the importance of this hormone in nodulation. On lateral roots, however, it has an inhibiting effect (Laplaze et al., 2007). Auxin efflux carrier PIN1, which is upregulated in lateral root formation, is readily removed from the plasma membrane of primordium cells upon cytokinin addition (Marhavy et al., 2011).

This paradox suggests that the mechanisms underlying auxin accumulation in lateral root and root nodule

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primordia differ, for which we offer additional experimental evidence. We hypothesize that the Nod-factor-activated cytokinin signaling causes local auxin accumulation in the cortical layers that form the nodule primordium.

We start with an unbiased analysis of three conceptually different mechanisms for local auxin accumulation. From this, we continue with the best candidate and discuss the likelihood that cytokinin can trigger the proposed mechanism.

To date, the formation of **lateral roots** has been studied most extensively in the model organism *Arabidopsis thaliana*. The lateral roots of these plants originate exclusively from a few pericycle cells, the "founder cells" (Malamy and Benfey, 1997; Casimiro et al., 2003). These cells are the site of the first auxin accumulation (Hirota et al., 2007). In model legumes, which, contrary to *Arabidopsis*, all have a multilayered cortex, the lateral root primordia are also predominantly of pericycle origin (Mallory et al., 1970), and the first auxin accumulation is observed in the pericycle (Rolfe et al., 1997; Larkin et al., 1996).

The formation of **root nodules** starts with an encounter of a compatible rhizobium bacterium, which is recognized by the plant host through the species-specific Nod factors it produces (Jones et al., 2007; see Chapter 51). Two major types of legume nodules are found on the model species: determinate and indeterminate, named after the persistence of a meristem only in the latter (Hirsch, 1992). One model legume, *Lotus japonicus*, forms determinate and the other, *Medicago truncatula*, forms indeterminate nodules. Interestingly, the primordia of determinate nodules originate in the outer to middle cortical layers, whereas the indeterminate primordia originate from the inner cortical layers (Hirsch, 1992). Correspondingly, local auxin accumulation has been found at the sites of the first cortical cell divisions in *Lotus* (Pacios-Bras et al., 2003; Takanashi et al., 2011) and white clover (indeterminate) (Mathesius et al., 1998).

Lateral roots and root nodules originate in approximately the same zone of the legume root. For nodules, this is called the susceptible zone (Bhuvaneswari et al., 1981). In the youngest part of this zone the epidermal cells start developing root hairs. Cortical cells in the zone differentiated fully and in the process of nodule formation they de- and redifferentiate. Throughout this text, we sometimes refer to the legume susceptible zone using "DZ" (standing for "differentiation zone"), thereby stressing the origin of the parameters we use (see Section 66.2).

Auxin is active in minute concentrations (Ljung et al., 2001; Marchant et al., 2002; Petersson et al., 2009). The most abundant natural auxin, indol-3-acetic acid (IAA) is a weak acid with $pKa \approx 4.8$. Its protonated form, IAAH, can passively cross the cell membrane, which is, however, practically impermeable to the deprotonated form, IAA^-. Because the apoplastic is slightly acidic and the cytoplasm neutral, this difference can result in an influx of auxin against the total auxin concentration (Mitchison 1980; Steinacher et al., 2012). This influx can be enhanced by more than an order of magnitude by auxin influx carriers such as the AUX1/LAX family (Swarup et al., 2005). These are typically located homogeneously over the cell membrane, or at increased levels at the apical and basal sides of the cell (Swarup et al., 2001; 2004, Kleine-Vehn et al., 2006). Efflux occurs predominantly by efflux carriers such as the PIN proteins (Galweiler et al., 1998; Paponov et al., 2005), often concentrated on specific sides of the cells. This gives rise to directional auxin transport (Benkova et al., 2003; Blilou et al., 2005). For an excellent review on auxin biosynthesis we refer the reader to Mano and Nemoto (2012). Because no tools exist yet for monitoring the auxin concentration live in living plants, computer models have become an indispensable tool in studying the many roles of this plant hormone in plant development – see (Kramer, 2008; Grieneisen and Scheres, 2009; Garnett et al., 2010; Jonsson and Krupinski, 2010) for some reviews.

From this overview of auxin transport and metabolism we arrive at three major scenarios for increasing the auxin concentration in a cell: increasing the amount of influx carriers, decreasing the amount of efflux carriers, and local auxin production (Deinum et al., 2012). We use a computational model of a generalized legume root segment to investigate the possibilities of inducing local auxin accumulation through these mechanisms and their distinguishing properties. For this we apply these scenarios to a designated part, the "**controlled area**" of an *in silico* root segment. We find that the three scenarios all yield different spatial and spatiotemporal signatures. Of these, the signature generated by a reduction of the efflux was most compatible with the observed patterns of auxin accumulation and the morphology of the nodule primordium. Because this differs from the mechanism of lateral root primordium initiation, we expect that root nodule positioning lacks the sensitivity to root curvature found in lateral roots, which we experimentally verify. The lateral position of the cortical auxin maximum can be shifted by small changes in the distribution of cortical PIN proteins. Based on this observation we discuss the possibility that cortical PINs determine the lateral position of the first cell divisions in nodulation, which, in turn, is correlated with the type of nodules produced.

66.2 METHODS

66.2.1 Simulations of Auxin Transport and Metabolism in Root Segments

Auxin transport and metabolism are modeled with subcellular precision. Within a cell and inside the apoplast, auxin

Table 66.1 Overview of model parameters (Deinum et al., 2012)

Parameter	(Default) Value	Description
D_c	300 μm²/s	Auxin diffusion constant inside cells (Laskowski et al., 2008)
D_W	44 μm²/s	Auxin diffusion constant in apoplast (Jones et al., 2009)
$P_{out,high}$	20 μm/s	Effective efflux permeability, high value (Grieneisen et al., 2007; Laskowski et al., 2008)
$P_{out,low}$	5 μm/s	Effective efflux permeability, low value (Grieneisen et al., 2007; Laskowski et al., 2008)
$P_{out,bg}$	1 μm/s	Effective efflux permeability, background value (due to misplaced PINs) (Grieneisen et al., 2007; Laskowski et al., 2008)
P_{in}	20 μm/s	Effective influx permeability (Grieneisen et al., 2007; Laskowski et al., 2008)
C_v	Concentration	The average auxin concentration in the vascular tissue is normalized to 1 C_v (Strictly speaking, C_v is not a parameter.)
p	0; 10^{-4} C_v/μm³/s	Auxin production rate; Default: no production. Estimates for reasonable rates based on (Ljung et al., 2001), scaled relative to total concentrations (Marchant et al., 2002)
l	100 μm	Cell length
w_C	20 μm	Width of cortical cells
w_x	10 μm	Width of other cells
d_W	0.2 μm	Wall thickness (Jones et al., 2009)
d_p	2 μm	Pixel size for the cells' interior
t	0.5, 1, 2.5 s	Integration time step (dependent on interval between measurements)

See Section 66.2 and (Deinum et al., 2012) for explanation.

moves by diffusion with diffusion constants D_C and D_W, respectively. Transport over the cell membrane is modeled using directional effective permeabilities P_{in} and P_{out} for influx and efflux. This implicitly assumes non-saturating processes or carriers operating far from saturation. This results in flux over the membrane (positive means outward flux): $J_{mem} = P_{out}\ C_c - P_{in}\ C_w$. In our simulations, efflux parameter P_{out} can be set for each cell face independently, whereas influx parameter P_{in} is always the same for all four faces of the cell. Parameters and default values are summarized in Table 66.1. Further details of the simulations can be found in Deinum et al. (2012).

Because of the lack of data on PIN positioning in the relevant root of a real model legume, the PIN layout was based on an experimentally founded model developed for *Arabidopsis* (Laskowski et al., 2008), geometrically adopted to resemble model legumes *Medicago* and *Lotus* (Deinum et al., 2012).

The simulation root segments we used are much longer than the region of interest, so as to minimize effects from the boundaries. Boundary conditions and motivation are further described in (Deinum et al., 2012).

66.2.2 Changes Applied in the "Controlled Area"

A designated part of the root segment (Fig. 66.1a and b), a 0.5 mm block comprising the epidermis and the cortex on one side of the root, is designated the "controlled area." All scenarios are implemented as a change applied to these cells: (n-fold) increase in the effective influx permeability, (n-fold) INFLUX, (n-fold) decrease of the effective efflux permeability, (n-fold) EFFLUX, and local auxin production with a given rate, PRODUCTION.

66.2.3 Concentrations are Normalized with Respect to the Vascular Tissue

For easier interpretation of the results we have normalized the concentration in the root segments with the average vascular concentration in a segment without any change in the controlled area. This level is called C_v. Thus, a concentration of $2C_v$ means twice as much auxin as the average for the vascular tissue. Where applicable, auxin production occurs with rate p in normalized concentration units per second per unit volume.

66.2.4 Plant Growth and Gravitational Stimulation

7–8 *M. truncatula* A17 seedlings were grown on each $24 \times 24\ cm^2$ plate with buffered Nod medium containing amino-ethoxy-vinyl-glycine (AVG). The gravitational field of the plants was changed by turning the plates by 90° at irregular 1–3 day intervals. Upon transfer to the plates, they were inoculated with rhizobium strain *Sinorhizobium meliloti* 2011 and covered by transparent sheets to ensure continued contact with the medium. The medium, seed treatment, and germination were according to standard protocols (www.noble.org/medicagohandbook).

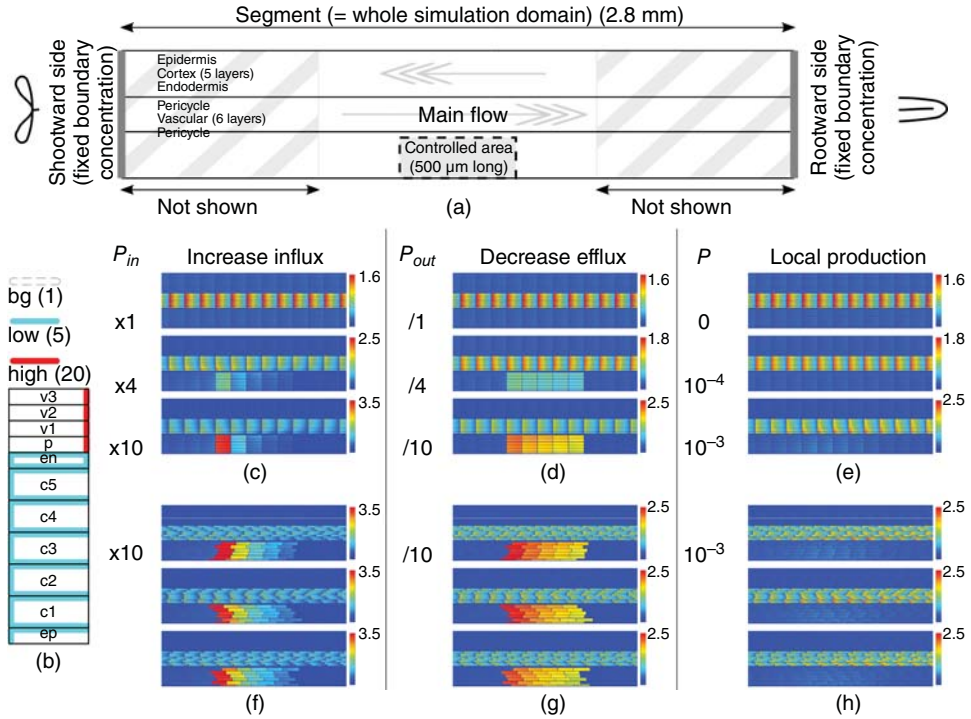

Figure 66.1 Different mechanisms for local auxin accumulation leave different signatures. General setup is a root segment representative of the susceptible zone of module legumes Lotus and Medicago (a). The distribution of PIN proteins (b) gives rise to net auxin fluxes as indicated in (a). PINs are modeled using effective efflux permeabilities P_{out} with three starting levels: "bg" (1 µm/s), "low" (5 µm/s), and "high" (20 µm/s), following (Laskowski et al., 2008). A five cells long block of cells, consisting of all five cortical layers and the epidermis is designated "*controlled area.*" Changes are applied to these cells: increase in the effective influx permeability, INFLUX (c,f), decrease in the effective efflux permeability, EFFLUX (d,g), or local auxin production, PRODUCTION (e,h), with indicated factors or rates. Note that INFLUX results in a much stronger accumulation on the right/downstream side of the controlled area, whereas EFFLUX results in more homogeneous accumulation. Randomly generated root segments (f–h) with an average cell length of 100 µm, normally distributed with $\sigma = 4$ µm, show that the observed patterns do not depend on the square layout of the default segments. Concentration ranges are adapted for maximum visibility, all ranging from 0 to the maximum (in C_v) indicated with each subfigure. Figure after Deinum et al. (2012).

66.2.5 Statistical Methods

All statistical calculations were made using self-written scripts. For comparison of fractions of lateral organs on curved vs. straight parts we calculated $p = \int_{q=0}^{1} P(f_L \leq \mathrm{q} \,\Lambda\, f_N = \mathrm{q})\mathrm{d}q$, with f_L and f_N representing the "true" fraction of lateral roots or nodules, respectively, on curved regions of the roots on a particular plate. Probability density functions for f_L and f_N were calculated based on binomial distributions: $P(f_L \leq \mathrm{q}) = \frac{1}{I_0} \int_{r=0}^{q} \binom{n}{c} r^c (1-r)^{n-c} \mathrm{d}r$, with c being the number of lateral roots on curved parts of the root out of a total of n observations, and I_0 being the total mass of the integral. With this statistic, $p = 0.5$ means that nodules and lateral roots are equally likely to appear in curved regions. As the fraction of root length that qualifies as being curved differs among plates, all comparisons were made within plates only.

66.3 RESULTS AND DISCUSSION

66.3.1 Different Mechanisms for Local Auxin Accumulation Leave Different Signatures

From the available knowledge on auxin transport and metabolism we have selected three conceptually different mechanisms that could in theory lead to local auxin accumulation: INFLUX, EFFLUX, and PRODUCTION (Deinum et al., 2012). We investigated the behavior of these mechanisms in an *in silico* root segment representative of the susceptible zone of a model legume (Fig. 66.1a and b). The PIN layout of this root segment results in a rootward auxin flux in the stele and a shootward flux in the cortex.

An increase in the effective influx permeability, INFLUX, resulted in an increase in the auxin concentration in the controlled area (Fig. 66.1c). This increase, however, was strongest on the left side of the controlled

area, which is its downstream side with respect to the cortical auxin flux. This strong focus on the downstream side occurred independent of the length of the controlled area (data not shown).

With a decrease in the effective influx permeability, EFFLUX, on the other hand, we observed a broad auxin accumulation pattern (Fig. 66.1d). Although the concentration on the shootward side of the controlled area was slightly higher than that on the rootward side, this bias was much weaker than with INFLUX.

With both INFLUX and EFFLUX, the minimal change for an increase in the maximum cortical concentration above the vascular level C_v was between 4-fold and 10-fold. Such levels were not reached with the local production scenario PRODUCTION (Fig. 66.1e). Regardless of the production rate, the concentration in the controlled area remained far below $1C_v$. This could not be attributed to a low production rate, as the highest rate we used should be considered high based on gas chromatography and mass spectroscopy measurements by Ljung et al. (2001) and Marchant et al. (2002). Moreover, the absolute increase in the vascular tissue rootward, that is, downstream, of the production site was higher than in the controlled area itself, demonstrating that the auxin was mostly transported away. This is in line with a proof-of-principle simulation (of the *Arabidopsis* root) by Grieneisen et al. in which the whole auxin maximum around the quiescent center (QC) of the root tip could be filled by a single auxin-producing cell, a distant cortical cell, that soon contained less auxin than cells near the QC and eventually contained almost the same concentration as the cortical cells surrounding it (Grieneisen et al., 2007).

These signatures did not rely on the regular alignment of the cell files and were robust against variations in cell length (Fig. 66.1f–h).

From the different signatures we can conclude that the accumulation mechanism affects the dimensions of the resulting primordium. The DR5 data of auxin accumulation in lateral root primordia by Hirota et al. (2007) actually show an auxin maximum that is more focused than the region where influx carrier AUX1 is upregulated.

The broad accumulation pattern found with the EFFLUX scenario seems most compatible with the experimental observation that nodule primordia are typically broader than lateral root primordia.

66.3.2 Lateral Roots and Nodules Respond Differently to Gravitropically Induced Curvature

On curved roots, lateral roots typically emerge on the convex side ("outside") of the curved part (Fortin et al., 1989). Moreover, lateral roots can be promoted by mechanical or gravitropical root bending (Fortin et al., 1989; Laskowski et al., 2008); the first induction method was also demonstrated in *M. truncatula* (Lillo, 2012). Computer simulations by Laskowski et al. show that the geometrical effect of the bending results in a slight increase in the local auxin accumulation, particularly in the pericycle. This can be enhanced by auxin-induced AUX1 expression, through a positive feedback between increased auxin accumulation and increased influx (Laskowski et al., 2008). If the initial auxin accumulation in root nodule primordia is indeed caused by a reduction of the efflux, we expect that they do not show the typical distribution with respect to root curvature as seen in lateral roots.

To investigate this, we subjected *M. truncatula* plants to irregular gravitropic stimuli to induce highly curved roots. Nodule and lateral root positions were scored in three categories with respect to curvature: convex, concave, and straight/unclear (Fig. 66.2). In line with observations from other species, and mechanical root bending experiments in *Medicago*, lateral roots showed a very strong preference

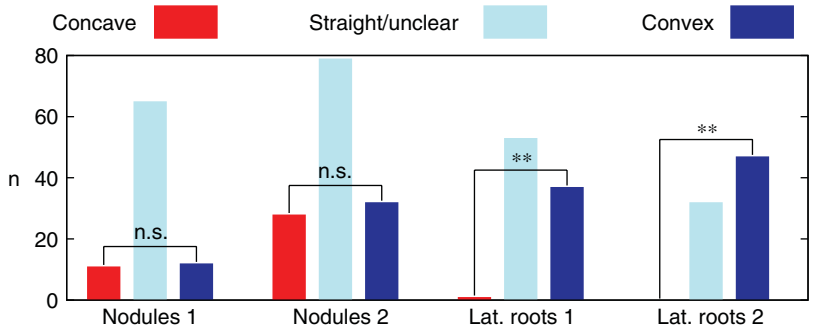

Figure 66.2 Root nodule position is insensitive to root curvature. Numbers of root nodules and lateral roots and their position with respect to root curvature: concave ("inside", red) or convex ("outside", blue) side of bend parts, or on straight parts (cyan) of the root. Root nodules showed no significant bias for either side of the root curve ($p = 1$, $p = 0.699$), whereas lateral roots showed a very strong bias toward the convex side ($p = 2.84\text{e-}10$, $p = 1.42\text{e-}14$). On both plates, lateral roots occurred relatively more often on bent regions than lateral roots (42% vs. 26%, $p = 0.014$ and 59% vs. 43%, $p = 0.011$). Roots were bent by exposing *Medicag truncatula* A17 plants on large square plates to varying gravitropic fields. Results of two representative plates with slightly different growth conditions and rotation regimes.

for the convex sides of roots ($p = 2.84\text{e-}10$, $p = 1.42\text{e-}14$, two-tailed binomial test). Root nodules, on the other hand, showed no significant bias for either the convex or the concave side of the curved parts of the root ($p = 1$, $p = 0.699$, two-tailed binomial test). Moreover, in both experiments, the percentage of lateral roots occurring in curved regions was larger than for root nodules on the same plants (42% vs. 26%, $p = 0.014$ and 59% vs. 43%, $p = 0.011$). This suggests that lateral roots are positively induced by root curvature, whereas nodules are not, or to a much lesser extent. Not only is this entirely in line with our expectation based on the different mechanisms, but it is also functionally relevant. While the average result of lateral root formation on the convex side of root curves rather than the concave side is a larger occupied soil volume, the main functional requirement for nodule primordia is that they originate close to the initial infection site and not on the other side of the vascular bundle if the root happens to be curved.

Based on our results so far we continued our investigation with a strong focus on the EFFLUX scenario.

66.3.3 Auxin Accumulation Following Efflux Reduction Starts Closest to Auxin Sources

In Figure 66.1 we investigated steady state patterns of auxin accumulation. A very relevant question is, therefore, whether the auxin accumulation occurs fast enough to be compatible with the known timing of nodulation events. In other words, a sufficient increase in auxin concentration is needed at least several hours prior to the first cortical cell divisions. This boils down to a time window of at most 20 h: at 18–24 h after inoculation, the cortical cells show cytoskeletal signs of activation for division (Timmers et al., 1999) and no cortical cell divisions are observed within 20 h after inoculation (Yang et al., 1994). As our approach involves an instantaneous change in the transport parameters and no transcription or translation of regulatory genes, the actual maximum time window is probably several hours shorter.

To check for compatibility, we followed the dynamics of auxin accumulation in the controlled area (Fig. 66.3). With our default parameters, the steady state was reached much faster than necessary (Fig. 66.3a and c). Auxin accumulation was also fast enough when we slowed down all dynamics by reducing all effective influx and efflux permeabilities by a factor 10 (Fig. 66.3b and d). Moreover, in the latter case enough time is left for processes we did not explicitly consider, such as the induction of the reduction of the efflux permeability and transcription of early nodulin genes. Such processes can happen fast, as most PIN1 disappears from the cell membrane in *Arabidopsis* lateral root primordia within 1 h upon cytokinin treatment (Marhavy et al., 2011) and early nodulin gene ENOD40 is upregulated within 3 h after spot inoculation in alfalfa (Compaan et al., 2001).

By studying the time scales of auxin accumulation under EFFLUX in different modified roots and different factors of efflux reduction, Deinum et al. found that the time scales of auxin accumulation are determined by the time scales of redistributing auxin over the whole controlled area. This, in turn, is largely set by the remaining effective efflux permeability at the shootward side of the cortical cells, that is, the side with most PINs, inside the controlled area (Deinum et al., 2012; see especially Fig. 66.3).

Interestingly, auxin accumulation after efflux reduction did not occur homogeneously throughout the controlled area. The first accumulation was seen in the innermost

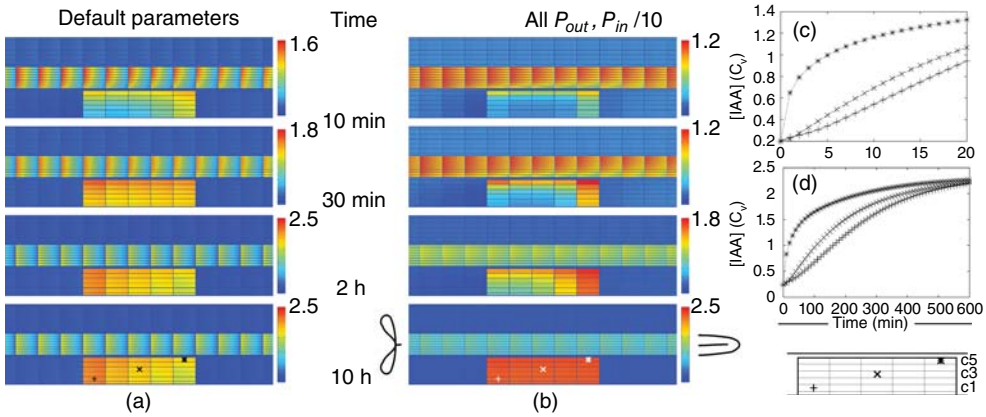

Figure 66.3 Auxin accumulation following an efflux reduction starts from the inner cortex, closest to the main auxin source. At T = 0 s, the efflux in the controlled area is reduced by a factor 10 (EFFLUX). The concentration is shown as heat maps at designated time points (a,b) and using time traces of the concentration in three designated cells in the controlled area (c,d). Both with default parameters (a,c) and with "slowed down" parameters (b,d), the auxin concentration increases first in the inner cortex and toward the left of the controlled area. These locations are closest to the vascular tissue and influx from unaffected cortex, respectively. "Slowed down": a 10-fold reduction of all influx and efflux parameters in the whole segment, prior to any change. Figure after Deinum et al. (2012).

cortical layer and also on the rootward (right) side of the controlled area (Fig. 66.3). These two locations correspond to the vicinity of auxin sources: the vascular tissue with its high auxin concentration and from the shootward flux in the cortex, respectively. The temporal difference was most pronounced with the reduced parameters (Fig. 66.3b and d; See also (Deinum et al., 2012), Fig. S2). As the auxin accumulation progressed, the transverse cortical concentration profile became flat again, just as in the root prior to induction.

66.3.4 Can Cortical PINs Make the Difference between "Indeterminate" (Inner Cortex) and "Determinate" (Middleouter Cortex) Auxin Accumulation?

A key difference between determinate and indeterminate is the radial position of the cortical cell divisions that form their primordia, and (most likely) the position of correlated auxin maximum. Determinate nodules originate from the middle to outer cortex, and indeterminate nodules from the inner cortex (Pacios-Bras et al., 2003; Takanashi et al., 2011; Mathesius et al., 1998; Libbenga and Harkes, 1973; Timmers et al., 1999). In our simulations so far, we assumed an equal amount of PIN proteins in the central and peripheral lateral membranes of the cortical cells. This resulted in an auxin maximum that was homogeneous in the radial direction (* in Fig. 66.4). We then investigated whether a central–peripheral bias in the effective efflux permeabilities could shift radial position of the EFFLUX auxin maximum and how strong such a bias would have to be.

For this we designed new reference segments with different inward and outward effective efflux permeabilities of the lateral cell membranes of all cortical cells and applied EFFLUX to all of those (Fig. 66.4). Already the smallest biases we tried, amounting to 20% more or 20% less outward than inward efflux, produced a marked radial shift of the auxin maximum in the controlled area, and even with the strongest inward bias the maximum concentration in the controlled area was well above the vascular concentration C_v.

Neither the formation of determinate nor the formation of indeterminate nodules defines a monophyletic group within the legumes (Doyle, 1994, Mergaert et al., 2003). This suggests that the transition between different nodules is relatively easy on evolutionary time scales. Moreover, grafting experiments show that the distinguishing feature must be root autonomous (Lohar and VandenBosch, 2005). Based on our results and the strict assumption that maximum auxin accumulation predicts the cell division site and through that nodule type, we tentatively hypothesize that slight changes in the lateral bias of PIN positioning could be such an "easy" change at the root of nodule type differentiation. A candidate system that could foster such changes would be the differential regulation of PINOID and/or PP2A activity, which plays an important role in the polar positioning of PIN proteins through their phosphorylation and dephosphorylation, respectively (Friml et al., 2004; Michniewicz et al., 2007). In a different context, changes in PINOID

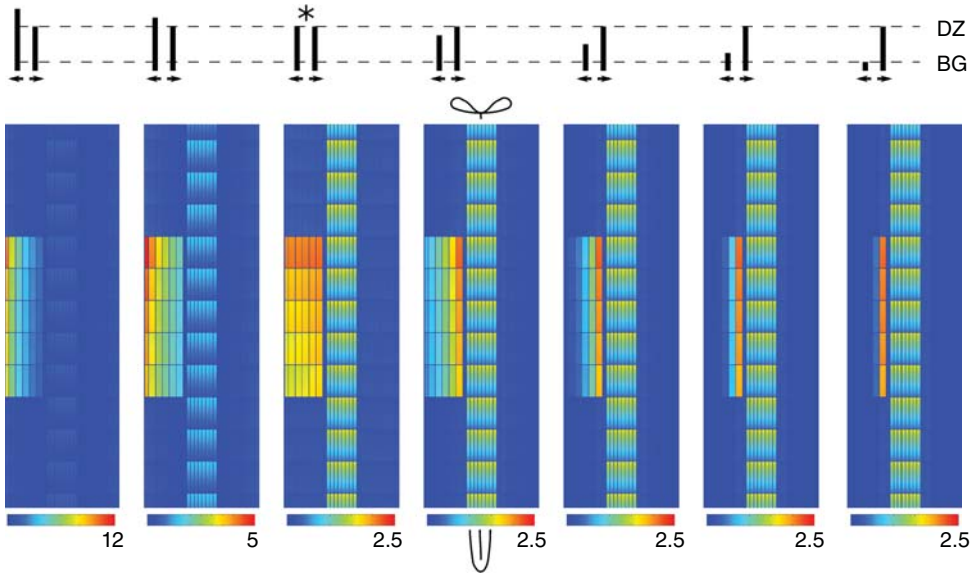

Figure 66.4 Changes in cortical PINs can shift the lateral location of the induced cortical auxin maximum. The maxima are all induced by the same decrease of efflux. The segments differ only in the effective efflux permeability of the outward faces of the cortical cell, ranging from 140% to 20% of the inward value, as indicated by the cartoon on top. The default layout, 100%, is indicated with *. In all cases, the highest concentration in the controlled area is above the vascular concentration C_v. Figure after Deinum et al. (2012).

activity affected the central–peripheral PIN distribution (Ding et al., 2011).

66.3.5 PIN Layout: Co-Determinant of the Susceptible Zone through Controlling Cortical Auxin Availability?

As the root develops, cells elongate and differentiate (Dello Ioio et al., 2008; Dolan et al., 1993) and also the distribution of PIN proteins over the different cell faces changes (Laskowski et al., 2008). In the original *Arabidopsis* model the root is divided into three major zones. Starting from the root tip, these are MZ ("meristematic zone"), EZ ("elongation zone"), and DZ (Laskowski et al., 2008). The susceptible zone for nodulation is most similar to the DZ with respect to geometry and developmental stage, as root hairs, the canonical rhizobial entry points, arise in the DZ. This made the DZ a very natural starting point for our simulation study. We wondered, however, whether other factors could also affect if and how easily lateral organs could be induced in particular root zones. We therefore investigated the effects of INFLUX and EFFLUX using the EZ PIN layout (Fig. 66.5i).

Interestingly, the same changes that produced large auxin accumulation in the default (DZ; Fig. 66.5a) segment (Fig. 66.5c and d) had apparently little effect in the EZ segment (Fig. 66.5k and l). Upon closer inspection, the radial concentration profiles in the controlled area were similar to those without any change (Fig. 66.5e–h). This shows that the propensity for auxin accumulation in the controlled area strongly depends on the cortical auxin availability, which is much larger in the DZ (Fig. 66.5b) than in the EZ (Fig. 66.5j) segment.

In the radial direction, the only difference between the EZ PIN layout – which shows markedly little auxin accumulation – and the rightmost segment of Figure 66.4 – which showed a strong auxin accumulation in the inner cortex – is in the endodermis. This suggests that a strong inward (or "L-shaped") PIN distribution in the endodermis could reinforce the distal boundary of the susceptible zone through a strong reduction in the amount of auxin available in the cortex. We like to add that the appearance and elongation of root

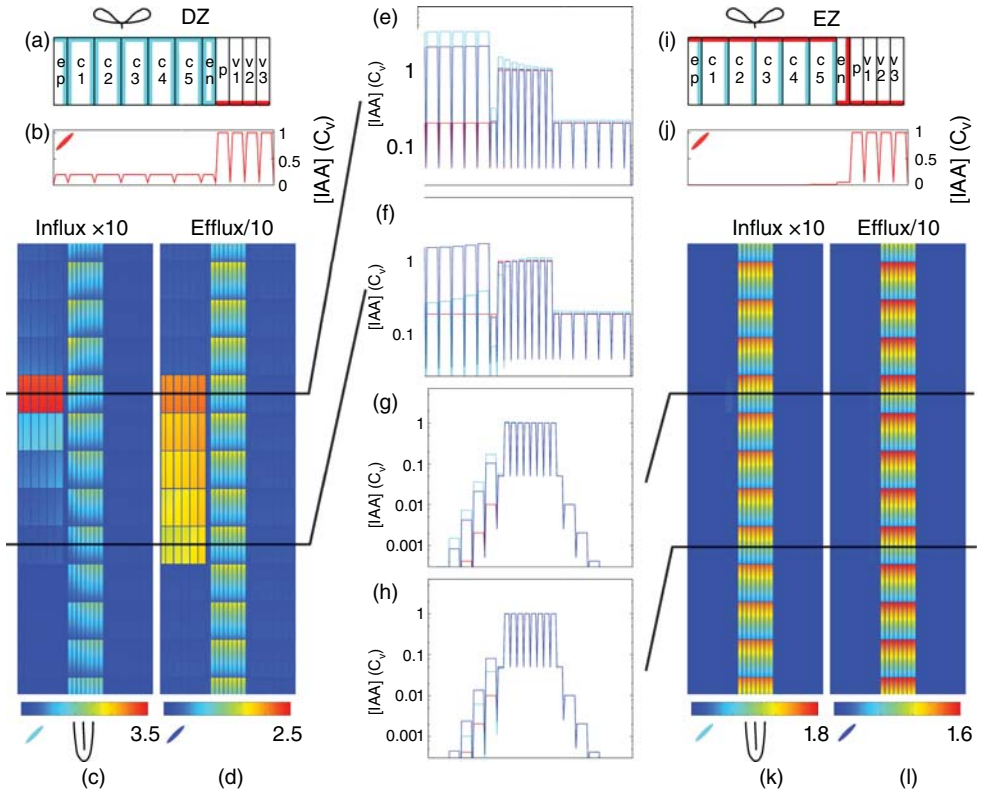

Figure 66.5 Local auxin accumulation in different zones of the root. The same changes, INFLUX and EFFLUX, are applied in different zones of the root, with strikingly different effects on auxin accumulation. The only difference between the root segments is the PIN layout (a,i), "DZ" (a–f) and "EZ" (g–l), as based on (Laskowski et al., 2008). The EZ PIN layout results in a much lower cortical auxin concentration (cf. j,b). Consequently, much less auxin accumulates in the controlled area after INFLUX (k vs. c) or EFFLUX (l vs. d) with the EZ layout. Transverse concentration profiles (e–h), plotted on a logarithmic scale for visibility, show that with both changes (INFLUX: cyan, EFFLUX: blue) the resulting profile in the controlled area resembles the original profile (red) in shape. Figure after Deinum et al. (2012).

hairs are also stimulated by auxin (Masucci and Schiefelbein, 1994; Pitts et al., 1998), so the same change of endodermal PIN positioning most likely has a double and congruent effect on specifying the susceptible zone in many legumes.

66.4 CONCLUSION AND OUTLOOK

To shed light on the earliest stages of root nodule formation and the role of auxin therein, we have investigated conceptually different mechanisms for local auxin accumulation in roots. These mechanisms all show their own characteristic signatures. Of the mechanisms investigated, a reduction of efflux was most compatible with diverse experimental observations. This is a different mechanism from the increased influx that is key to lateral root formation, predicting a different sensitivity of lateral root and root nodule formation to root curvature, which we subsequently observed in *Medicago* plants upon gravitropic stimulation. The simple approach taken here, of course, has its limitations, which marks the mechanism of their induction as an important topic for follow-up investigations. Further computational studies could also help identify downstream consequences of our tentative hypothesis on different PIN distributions in relation to nodule type that are easier to address experimentally.

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Section 11

Transitions from the Bacterial to the Bacteroid State

Chapter 67

Bacteroid Differentiation in Legume Nodules: Role of AMP-Like Host Peptides in the Control of the Endosymbiont

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67.1 INTRODUCTION

Despite constituting 78% of the atmospheric air, nitrogen is the most limiting nutrient in the soil for plant growth. This is compensated in agriculture with the extensive use of nitrogen fertilizers. Many leguminous plant species can, however, overcome this nutrient limitation by forming symbiosis with nitrogen-fixing α- and β-proteobacteria collectively known as rhizobia. Legume plants select their bacterial partners from the rhizosphere via molecular signaling. Flavonoids excreted from the host root induce in proper rhizobia the expression of nodulation genes required for the production of host-specific bacterial lipochitooligosaccharide signals called Nod factors (Schultze et al., 1994; see Chapters 50, 51). These molecules induce the formation of a specific symbiotic organ, the root nodule where bacteria in the nodule cells are converted into nitrogen-fixing bacteroids and reduce the atmospheric molecular nitrogen to ammonia. The symbiosis is mutually beneficial as the endosymbionts provide ammonia to the host for plant growth in return for photosynthetic products (carbon source and energy).

Bacterial invasion occurs generally via the root hairs: the bacteria attach to the tip of growing root hairs that curl around them and then the entrapped bacteria enter the root

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hair cell through invagination of plasma membrane and formation of a tube-like structure, the infection thread (IT). ITs can cross cell boundaries and transport the bacteria toward the root cortex. Simultaneously with the infection cortical cells start to divide, leading to the development of the nodule primordium. When the growing ITs reach the nodule primordium, nodule differentiation begins. The bacteria are released from the ITs into the cytoplasm of nodule cells via an endocytosis-like mechanism, and as a result the rhizobia are encapsulated in a plant-derived peribacteroid membrane and exist as an organelle-like structure called symbiosome. After infection, the nodule cells start to grow mostly by repeated genome doublings via endoreduplication (ER) cycles until they are large enough to accommodate a multitude of bacteroids. Nitrogen-fixing bacteroids are differentiated rhizobia whose metabolic activity is adapted to the intracellular life style, the specific requirements for nitrogen fixation, and exchange of metabolites between the partners (White et al., 2007).

Despite the common, nitrogen-fixing function of bacteroids, the morphology, physiology, and differentiation fate of bacteroids can be strikingly diverse in the different legume nodules. Certain rhizobia can switch between the free-living and the endosymbiotic life styles while for others the symbiotic state cannot be reversed to the free-living one; thus, their fate is irreversible and terminal. In this review, the two major nodule types in relation to reversible and terminal endosymbiont differentiation are presented as well as the current knowledge on the plant effectors of terminal bacteroid differentiation.

67.2 TWO NODULE TYPES WITH COMMON FEATURES OF SYMBIOTIC HOST CELLS AND DIFFERENT FATE OF ENDOSYMBIONTS

The form and size of nodules can be quite different in the various legume hosts. Nevertheless, they can be classified either as determinate or indeterminate type (Fig. 67.1a). The nodule type depends on the host plant and frequently reflects taxonomical position (Sprent, 2007). The major difference between the determinate and indeterminate nodules is the transient or persistent nature of the nodule meristem, respectively. In addition, determinate nodules originate from division of the outer cortical cells while the indeterminate ones originate from the inner cortical cells. Determinate nodules are spherical since cell division is only maintained at the primordium stage and later, while the cell number does not increase, the nodule growth is achieved by cell growth. In the indeterminate nodules, the cell number increases as the persistent apical meristem produces permanently new nodule cells, which together with cell enlargement result in continuous nodule growth and a cylindrical nodule shape. Determinate nodules are formed on (sub-)tropical plants such as *Phaseolus*, *Glycine,* and *Vigna* species belonging to the phaseolids or on robinioids such as *Lotus* and *Sesbania* species; and dalbergioids such as *Arachis* and *Aeschynomene* species. Indeterminate nodules develop on temperate legumes, such as *Medicago truncatula, Pisum sativum, Vicia faba, Trifolium repens,* or *Galega orientalis,* which all belong to the inverted repeat-lacking clade (IRLC) but indeterminate nodules can be found in almost all major legume clades.

In the determinate nodules, the infected cells develop simultaneously and therefore the host symbiotic cells are always in the same developmental stage. The central part of mature determinate nodules is composed of a mixture of uninfected cells and large symbiotic cells packed with nitrogen-fixing bacteroids (Fig. 67.1b). The structure of indeterminate nodules is more complex (Fig. 67.1b) and an age gradient and all stages of symbiotic cell development are present in the nitrogen-fixing nodules. The central region of indeterminate nodules contains the apical meristem (zone I], the infection zone [zone II], an interzone II-III, the nitrogen fixation zone [zone III], and in old nodules a senescent zone [zone IV] (Vasse et al., 1990). Infection of plant cells with *Rhizobium* bacteria occurs in the non-dividing post-meristematic cells, which enter a multistep differentiation program along the 10–15 cell layers of zone II. It is manifested by gradual enlargement of the symbiotic nodule cells reaching an about 80-fold larger volume than the meristematic cells. It was shown in *M. sativa* and *M. truncatula* that growth of host symbiotic cells arises from repeated ER cycles: doubling the genome in each cycle along the different cell layers of zone II and resulting from diploid (2C or 4C) meristematic cells in nitrogen-fixing cells with 32C or 64C DNA content (Fig. 67.1b). Studying host symbiotic cell development resulted in the discovery of the cell cycle switch protein CCS52A, which is a key regulator of cell differentiation in plants controlling cell cycle exit, transition of mitotic cycles to ER cycles (termed also endocycles), and the execution of multiple rounds of ER cycles (Cebolla et al., 1999). ER is an essential part of host symbiotic cell development; if the 32C/64C ploidy levels are not completed, nodule development becomes aborted leading to early nodule senescence and rapid disintegration of bacteroids (Vinardell et al., 2003). Though the nodule ploidy levels show variations in the different legumes, it is generally valid that symbiotic nodule cells are large and usually polyploid, which may be necessary for hosting several tens of thousands of rhizobia in the host cytoplasm as well as for altered physiology of these cells.

The morphology of nitrogen-fixing bacteroids can be strikingly different. Three morphotypes are known: U-, S-, and E-morphotypes. The U-type corresponds to bacteroids with unaltered/undifferentiated morphology compared

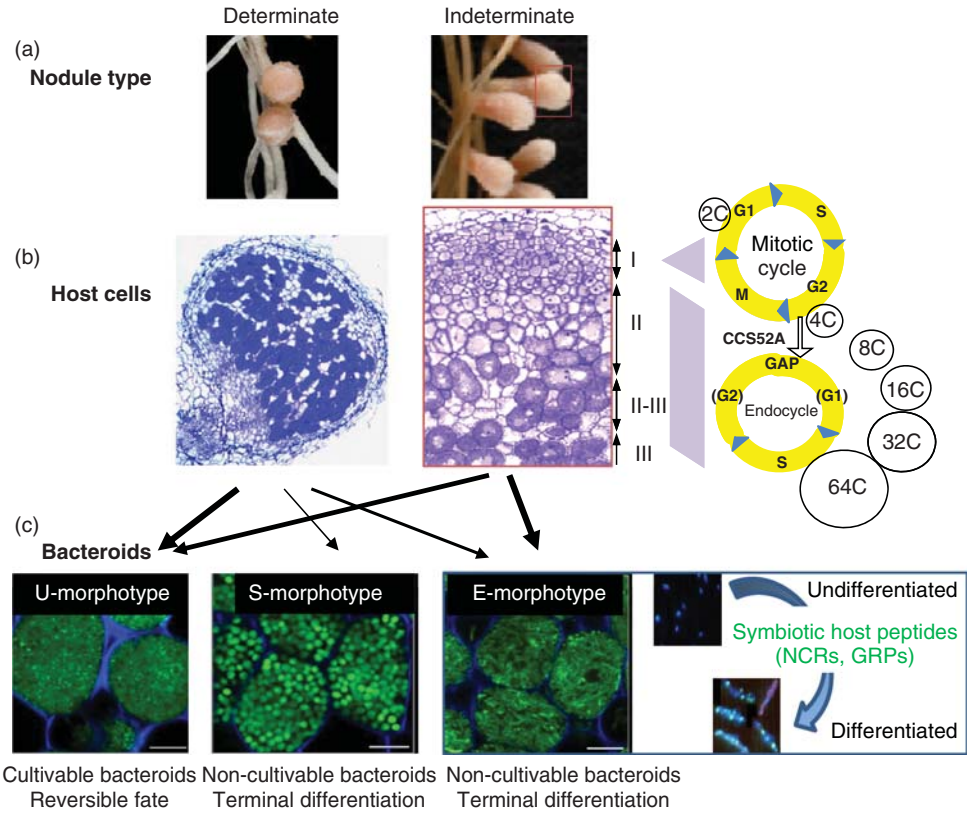

Figure 67.1 Differentiation of host symbiotic cells in legume root nodules. (a) Morphology of the two nodule types. Determinate nodules are spherical while the continuously growing indeterminate nodules are cylindrical. (b) Symbiotic cells in nitrogen-fixing root nodules are large, usually polyploid and filled with nitrogen-fixing bacteroids. The central zone of determinate nodules corresponds to the nitrogen-fixing zone [III], which contains large nitrogen-fixing plant symbiotic cells [dark blue] and smaller uninfected nodule cells. In contrast, the indeterminate nodules are composed of different nodule zones. The longitudinal nodule section shows the apical nodule region [red frame] with I: nodule meristem, II: infection zone, II-III: interzone, III: nitrogen fixation zone [only the first cell layer is visible on the section]. In zone II and interzone II-III gradual enlargement of host symbiotic cells is governed by a switch of the mitotic cycle to endoreduplication cycles, which is induced by CCS52A, resulting in the development of large polyploid host symbiotic cells. (c) Different morphology of bacteroids is host but not nodule-type dependent. U-morphotype bacteroids are similar to free-living bacteria and by maintaining their reproductive capacity they can return to the free-living life. S-morphotype bacteroids are swollen, spherical, and non-cultivable. This bacteroid morphology is rare among the tested legumes. E-morphotype bacteroids are elongated and often branched Y-shaped cells that cannot be cultivated. Thus, the S- and E-morphotype bacteroid differentiation is terminal. All three morphotypes were found in determinate nodules while indeterminate nodules were so far associated with U- and E-morphotype bacteroids. In the IRLC legumes E-morphotype bacteroid differentiation is achieved by host-specific symbiotic peptides [NCRs and possibly GRPs] that likely evolved from antimicrobial peptides of the innate immunity. Examples are *Bradyrhizobium japonicum* U-morphotype bacteroids in soybean, *Bradyrhizobium* strain ORS285 S-morphotype bacteroids in *A. indica,* and *Bradyrhizobium* strain ORS285 E-morphotype bacteroids in *A. afraspera*.

to their free-living siblings. The symbiotic life style of these bacteroids is reversible; bacteroids isolated from the nodules can be cultivated and return to the free-living state. S-morphotype corresponds to large spherical bacteroids, while the E-morphotypes correspond to large elongated and often Y-shaped bacteroids. Neither the S- nor the E-morphotypes are cultivable; thus, their differentiation fate is one way, terminal. The bacteroid morphotype is not a consequence of the nodule type. Bacteroids in the indeterminate nodules can be U- or E-morphotype or could be U-, S-, or E-morphotype in the determinate nodules (Fig. 67.1). Thus, the differentiation fate of bacteroids does not depend on the nodule type but it nevertheless depends on the host plant. For example, in *M. truncatula* nodules the bacteroids undergo always a terminal E-morphotype differentiation process while in *Lotus japonicus*, *Phaseolus vulgaris, Glycine max,* or *Sesbania rostrata*, bacteroids are of the reversible U-type. E-morphotype is common in the IRLC legumes such as *P. sativum*, *V. faba, T. repens,* or *G. orientalis* or the cultivated alfalfa *M. sativa* and any other *Medicago* species. S-type bacteroids can be found in *Arachis* species and in certain *Aeschynomene* and *Crotalaria* species. While 12,000

legume species are known to form nitrogen-fixing symbiosis, only a few of them have been studied with respect to the morphology and physiology of bacteroids. Recently, 40 legume species belonging to 6 subclasses in the *Papilionoideae* subfamily were examined and members of 5 subclasses contained terminally differentiated bacteroids (Oono et al., 2010). The terminally differentiated, non-reproductive bacteroids were proposed to be more effective in nitrogen fixation than the reversibly differentiated, reproductive ones. One possible explanation for the difference in effectiveness can be that part of the plant-provided metabolites are used in reversibly differentiated bacteroids for the production of storage compounds such as polyhydroxybutyrate (PHB), and therefore these endosymbionts require more nutrients from the plant than the terminally differentiated ones to provide the same amount of combined nitrogen. Thus, the terminal bacteroid differentiation might be a host benefit that has evolved independently with multiple origins in the *Leguminosae* family (Oono et al., 2010).

The morphological diversity of bacteroids has been known since the end of the nineteenth century but the molecular mechanisms leading to terminal bacteroid differentiation were uncovered only a few years ago in the *S. meliloti–M. truncatula* symbiosis (Mergaert et al., 2006). Nitrogen-fixing *S. meliloti* bacteroids are 5- to 10-fold longer than the free-living bacteria and are often branched, having a characteristic Y shape. The bacteroids are polyploid cells harboring multiple nucleoids. Genome amplification up to 24C DNA content is achieved by ER cycles and inhibition of cell division. All these changes result in the definitive loss of the bacteroids' reproductive capacity. In addition, the bacteroid membrane is more permeable than the bacterial membranes since propidium-iodide, a dye that cannot enter into intact cells, penetrates slowly into the isolated bacteroids. In symbiosis, the increased membrane permeability of bacteroids may be advantageous, facilitating the exchange of metabolites between the host and endosymbionts (see also below).

Terminal bacteroid differentiation is host controlled and driven by plant factors present in IRLC and missing from non-IRLC plants (Mergaert et al., 2006). It was shown that the fate of the same *Rhizobium* strains was different in the IRLC- and non-IRLC legume hosts; bacteroids in the IRLC-nodules were terminally differentiated with E-morphotype and with reversible U-morphotype in non-IRLC nodules. Similarly, *Bradyrhizobium sp. 32H1* bacteroids in peanut nodules were terminally differentiated with S-morphotype while in cowpeas it was the reversible U-morphotype (Oono and Denison, 2010). Another example is *Bradyrhizobium* strain ORS285, which forms S-morphotype bacteroids in nodules of *Aeschynomene indica* and E-morphotype bacteroids in nodules of *Aeschynomene afraspera* (Fig. 67.1) (Bonaldi et al., 2011; see Chapters 28, 45).

67.3 IDENTIFICATION OF KEY PLANT FACTORS MEDIATING TERMINAL BACTEROID DIFFERENTIATION

Discovery of plant factors responsible for the terminal bacteroid fate in the IRLC legumes was based on the presumption that the plant factors (i) are specific for the IRLC species, (ii) are produced in the infected (cells of) nodules, (iii) are targeted to the bacteroids, (iv) change the permeability of bacterial membranes, and (v) affect bacterial cell division. First, global transcript profiling of *M. truncatula* and *L. japonicus* nodules (containing terminally and non-terminally differentiated bacteroids, respectively) was used to identify IRLC-specific genes. In this way, two large gene families coding for nodule-specific cysteine-rich (NCR) and glycine-rich peptides (GRPs) were recognized in *M. truncatula* (Kevei et al., 2002; Mergaert et al., 2003). Similar sequences were found among the nodule-specific transcripts of other IRLC legumes such as *P. sativum*, *V. faba*, *G. orientalis,* and *Astragalus sinicus* (Scheres et al., 1990; Küster et al., 1995; Schröder et al., 1997; Frühling et al., 2000a; Kaijalainen et al., 2002; Chou et al., 2006), but no *NCR* and *GRP* genes could be revealed in the sequenced genomes of *L. japonicus* and *G. max* (Alunni et al., 2007).

So far, 24 members of the GRP family are known in *M. truncatula* that can be divided into six subfamilies containing 13 cationic, 8 anionic, and 3 neutral peptides (Kevei et al., 2002, Alunni et al., 2007). GRPs are composed of a relatively conserved signal peptide and a mature peptide of mostly ~100 amino acids. GRPs are expressed in the plant symbiotic cells but the different members are expressed at distinct stages of development; therefore, they are potential bacterial differentiation factors; however, their functions and their proposed roles in bacteroid differentiation remain to be discovered.

At present, about 600 members of the NCR family are known (Young et al., 2011). The *NCR* genes are scattered on the eight chromosomes of *M. truncatula* but often they are clustered. The *NCR* genes are small and usually consist of two exons: the first one codes for a relatively conserved signal peptide while the second one codes for the mature, usually 30–50 amino acid long, active peptide. The NCR peptides are highly divergent but all of them contain four or six cysteines in conserved positions (Mergaert et al., 2003). As a consequence of diverse amino acid composition, the isoelectric point [pI] of the NCR peptides is remarkably different; thus, the peptides can be anionic, neutral, or cationic.

Promoter–reporter gene fusion studies as well as microarray and *in situ* hybridization experiments (see in more detail in the next paragraph and Fig. 67.2) revealed that the *NCR* and *GRP* genes are expressed exclusively in

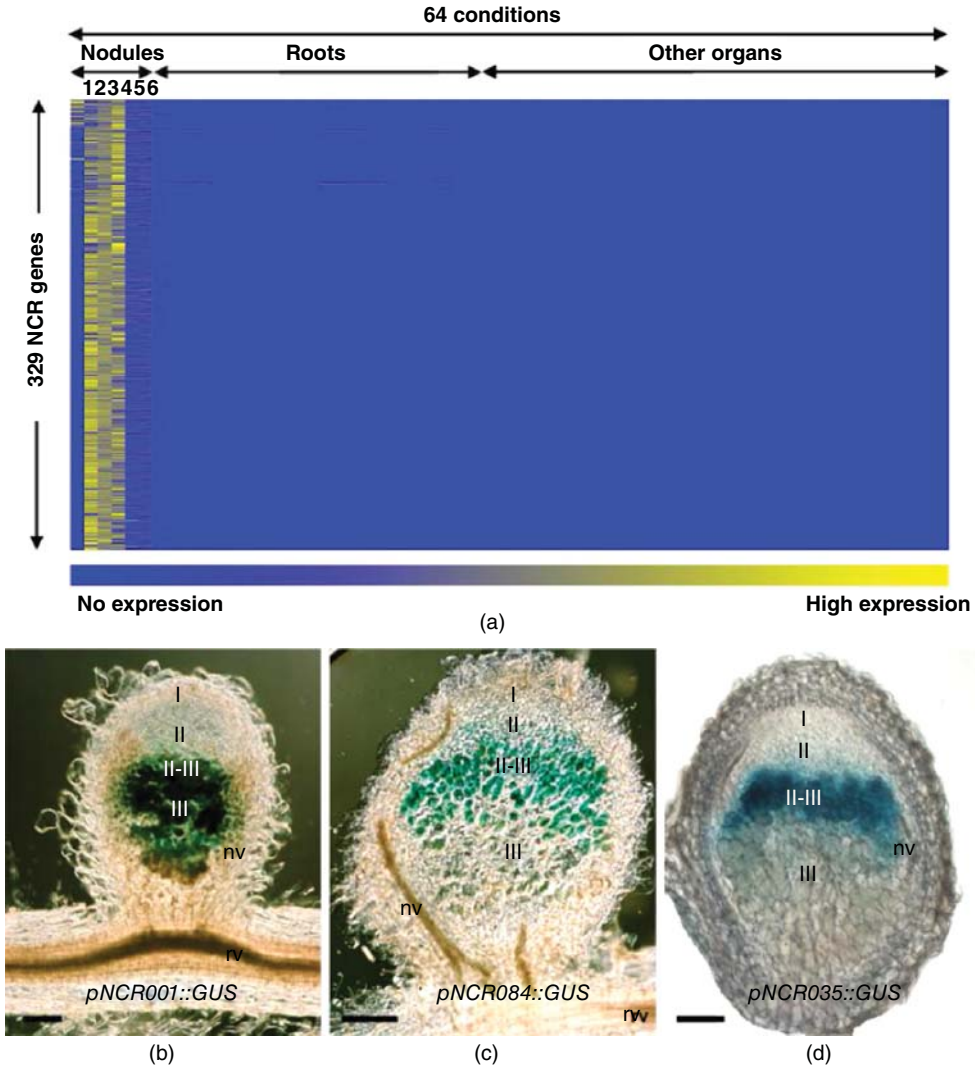

Figure 67.2 Expression pattern of NCR genes. (a) The heat map displays the expression level of 329 NCR genes over 64 different experimental conditions. The data were extracted from the MtGEA. The experiments are divided into "nodules," "roots," and "other organs." The nodules are, in the order from 1 to 6, those at 4 days post infection [dpi], at 10 dpi, 14 dpi, 28 dpi, 14 dpi plus 2 days of nitrate treatment, and whole roots carrying nodules. The roots conditions included control roots, roots infected with mycorrhiza and *Phymatotrichipsis,* and salt-stressed roots. The other organ conditions included leaves and phytohormone-treated leaves, flowers, seed pods, seeds at different stages of formation, shoots, stems, buds, and cell suspensions treated with elicitor or methyl jasmonate. (b–d) Examples of NCR promoter-GUS fusions showing expression in symbiotic nodule cells. NCR001 (b) is expressed in the nitrogen-fixing host symbiotic cells while NCR084 (c) and NCR035 (d) are expressed in young cells. Scale bars are 200 µm. nv is nodule vasculature and rv is root vasculature. The location of the nodule zones I [meristem], infection and differentiation zone [II], interzone [II-III] and nitrogen-fixation zone [III] are indicated.

the root nodules and the expression is associated with the bacterial invasion of the plant cells (Küster et al., 1995; Mergaert et al., 2003; Kevei et al., 2002; Maunoury et al., 2010; Nallu et al., 2013). The signal sequence directs the passage of proteins and peptides through the secretory pathway to their destination. The functionality of the NCR signal peptides was proven in onion epidermal cells (Mergaert et al., 2003). In nodules, the NCR035-mCherry fusion protein co-localized with bacteroids, demonstrating that the peptides are targeted to the endosymbionts (Van de Velde et al., 2010). Generally, the cationic NCRs interacted with the bacterial membranes but certain peptides such as the anionic NCR001 was found by immunogold transmission electron microscopy in the cytosol of bacteroids. Moreover, several NCRs were detected by mass spectrometry in protein extracts of bacteroids isolated from *M. truncatula* nodules (Van de Velde et al., 2010). More recently, the development of proteomics techniques has allowed the identification of ~200 NCR peptides in bacteroids (Dürgő et al., personal communication).

Targeting of the peptides to the endosymbionts depends on the presence of a functional secretory pathway in the plant. The *M. truncatula dnf1-1*(defective in nitrogen fixation) mutant is impaired in a nodule-specific subunit of the signal peptidase complex [SPC, Wang et al., 2010] and forms small white nodules that fail to fix nitrogen. SPC is present in the endoplasmic reticulum (ER) where it can remove the signal peptide from nascent secreted proteins, allowing their proper targeting via the secretory pathway to their specific destination. In the *dnf1-1* mutant, formation of ITs and release of bacteria into host cells are normal but rhizobia remain undifferentiated (Wang et al., 2010; Van de Velde et al., 2010). In the *dnf1-1* mutant, the NCR peptides remain unprocessed since the defective SPC cannot remove the signal peptide from the NCRs. These unprocessed peptides are trapped in the ER and cannot interact with the bacteroids. This has proven that (i) direct interaction of NCRs with bacteroids is essential and that (ii) NCRs (and possibly the GRPs as well) are plant factors of terminal bacteroid differentiation.

Cationic NCR peptides are able to alter the permeability of the bacterial cell membranes. When peptides were administered to cultured bacteria, the membrane impermeable dye, propidium iodide was able to penetrate the bacterial membranes. *In vitro* treatment of bacteria with peptides induced also other features of bacteroid differentiation, such as cell enlargement and an increase of the DNA content in *S. meliloti* cells (Van de Velde et al., 2010; Haag et al., 2011).

The hypothesis that NCRs can govern the terminal bacteroid differentiation was further supported by the expression of NCRs in *Lotus japonicus* nodules (naturally lacking NCRs) under the control of the *M. truncatula leghemoglobin-1 promoter (pMtLb1)*. In control transgenic nodules expressing β-glucuronidase (GUS), several small undifferentiated U-morphotype bacteroids were present within the symbiosome as in wild type *L. japonicus* nodules. In contrast, upon expression of *pMtLb1::NCR035* in transgenic nodules, the majority of symbiosomes contained a single, frequently elongated bacteroid indicating that bacteria exposed to NCR035 have lost their reproductive capacity (Van de Velde et al., 2010). These data indicate that the expression of NCR peptides could provoke features of terminal E-morphotype bacteroid differentiation in the non-IRLC legume *L. japonicus.* Thus, taken together, the *in vitro* and *in vivo* evidence demonstrates that the NCR peptides are host mediators or effectors of the initiation of differentiation of E-morphotype bacteroids.

67.4 GENE EXPRESSION ANALYSIS OF NCRs

Besides the size of the NCR family in *M. truncatula* (600 genes), another surprising feature of the family is the (nearly) exclusive expression of the genes in nodules. The expression of NCR genes has been studied – mostly in *M. truncatula* but also in some other IRLC legumes – at the level of individual genes by RT-PCR, *in situ* hybridization, immuno-localization, and promoter–marker gene fusions (Scheres et al., 1990; Kardailsky et al., 1993; Frühling et al., 2000b; Kato et al., 2002; Crockard et al., 2002; Mergaert et al., 2003; Alunni et al., 2007; Van de Velde et al., 2010; Nallu et al., 2013) or at the family level by expressed sequence tag (EST) analysis, macroarrays, dedicated microarrays, or whole-genome microarrays (Fedorova et al., 2002; Mergaert et al., 2003; Graham et al., 2004; Maunoury et al., 2010; Moreau et al., 2011; Nallu et al., 2013; Tesfaye et al., 2013). Moreover, the *Medicago truncatula* Gene Expression Atlas (MtGEA) (Benedito et al., 2008; He et al., 2009), which compiles whole genome microarray data over a large set of experiments, offers a unique resource for analyzing the expression of *NCR* genes (Fig. 67.2).

Perhaps the most surprising finding revealed by these transcription studies collectively is that the complete *NCR* gene family, with only two exceptions, is specifically expressed in nodules. For example, analysis of 120,000 ESTs in 26 different cDNA libraries that covered most plant organs as well as different growth conditions including biotic and abiotic stresses demonstrated that ESTs of the 311 analyzed NCRs were only found in nodule libraries except for NCR122 and NCR218 (Mergaert et al., 2003). In addition, this analysis revealed that the NCR transcripts constitute almost 5% of the total mRNA population in nodules. A more recent study assessing the expression of more than 500 *NCR* genes with dedicated Affymetrix chips confirmed the nodule specificity of the *NCR* gene family (Nallu et al., 2013; Tesfaye et al., 2013). Currently the richest resource for gene expression analysis in *M. truncatula* is provided by MtGEA. MtGEA is a compendium of expression profiles for the majority of *M. truncatula* genes and is generated with the whole genome Affymetrix Medicago Gene Chip. The compendium covers the plant's major organs, various kinds of abiotic and biotic stresses, and data from specific cell and tissue types (Benedito et al., 2008; He et al., 2009). The expression pattern of 329 *NCR* genes, in 64 different experimental conditions, is shown in Figure 67.2a. This analysis confirms that NCRs are not expressed in any plant organ other than nodules. Only NCR122 and NCR218 are expressed also in roots, confirming the EST analysis. Moreover, it shows that the *NCR* genes are not induced by other infections such as with the pathogenic fungus *Phymatotrichopsis omnivore* or symbiotic mycorrhiza. Likewise, phytohormones, elicitors, and abiotic stresses such as salt or nitrogen starvation do not activate the genes.

In situ detection of *NCR* expression by *in situ* hybridization, immune-localization, or promoter-GUS/GFP/mCherry analysis has demonstrated for all the tested genes that they are expressed in the host symbiotic cells but different subsets

of *NCR* [as well as *GRP*] genes are activated at different stages of differentiation of these host cells (Fig. 67.2b–d) (Scheres et al., 1990; Kardailsky et al., 1993; Frühling et al., 2000b; Kato et al., 2002; Crockard et al., 2002; Mergaert et al., 2003; Van de Velde et al., 2010; Nallu et al., 2013). Transcriptome analysis with microarrays or Affymetrix chips extended this pattern to the whole family. During development of wild type nodules, the *NCR* genes are activated in consecutive waves and their first appearance coincides with the formation of host symbiotic cells (Fig. 67.2) (Maunoury et al., 2010; Nallu et al., 2013). Moreover, the pattern of *NCR* expression in bacterial or plant symbiotic mutants, forming non-functional nodules and arrested at different stages of nodule development, is also in agreement with a specific expression in the symbiotic nodule cells. For example, in nodules of the *M. truncatula* TE7 mutant, which is affected in the *IPD3* gene (Horváth et al., 2011; Ovchinnikova et al., 2011), and in nodules of the *S. meliloti exoY* mutant no host symbiotic cells are formed and these nodules do not express any of the *NCR* genes. In the *S. meliloti bacA* mutant on the contrary, a subset of *NCR* genes is activated (see Chapter 31) while in other mutants, forming normal host symbiotic cells with differentiated bacteroids, *NCR* genes are activated to a similar extent as in the wild type (Maunoury et al., 2010; Nallu et al., 2013).

Very little is known about how the very specific regulation of the NCRs is achieved. The transcription factor EFD, belonging to the ethylene response factor family, may control, directly or indirectly, the expression of a subset of *NCR* genes since in the *efd* mutant nodules, part of the *NCR* genes are downregulated and bacteroid differentiation is partially impaired (Vernié et al., 2008). Searching in the promoters of *NCR* genes with different algorithms yielded five different conserved motifs of 41 to 50 bp that are specifically enriched in the 1000 bp promoter regions (Nallu et al., 2013). Some of these motifs show resemblance to previously described motifs that were found in soybean and *Sesbania rostrata* leghemoglobin promoters and confer symbiotic cell-specific expression (Jensen et al., 1988; Szczyglowski et al., 1994). However, the implication of these motifs in the remarkable expression pattern of the *NCR* genes needs further investigations.

67.5 POSSIBLE FUNCTIONS AND TARGETS OF NCRs

The *M. truncatula* genome has the coding potential for close to 600 NCR peptides that are highly divergent in amino acid composition and in isoelectric point (ranging from 3.2 to 11.25, with approximately equal number of anionic and cationic peptides). Generally, in nature, one or only a few proteins is/are dedicated for a given biochemical function. In large protein families, individual members can have unique specificities such as the receptor families where closely related polypeptides discriminate between different signals. Thus, it is conceivable that the NCR family is not required for a single function, but rather that it provides multiple activities during bacteroid differentiation. It can be that distinct sets of peptides act in a concerted manner at different stages. This idea is supported by the different spatial and temporal gene expression patterns of *NCR* genes (Fig. 67.2b–d): some being induced in the youngest while others in the older cells of zone II, or specifically in the interzone II-III or in zone III indicating their involvement from the earliest stage, from multiplication of the symbiosomes after their release from ITs until the later and latest developmental steps. Diversity of NCRs arises from gene duplications, the recent ones with more homologous sequences than the ancient ones. It is likely that of the 600 NCR peptides, many would have redundant functions; however, we cannot exclude the possibility that master NCRs might exist whose function is unique and cannot be substituted by any of the 600.

The major function of some nodule-specific peptides might be the inhibition of bacterial cytokinesis after symbiosome multiplication ceases, which – while DNA replication is ongoing – results in the high DNA content and cell size of rhizobia and the formation of symbiosomes containing a single bacteroid surrounded by the peribacteroid membrane. This might be achieved either by inhibiting, for example, the interaction between FtsZ monomers and thus, the assembly of the Z ring, or by affecting the structure of bacterial membranes. Other peptides might be responsible for the maintenance of the ER cycles until bacteroids reach their final size. Another group of peptides can be responsible for the membrane modifications including changes in the permeability that can be observed in the bacteroids of IRLC legume nodules. In zone III, the peptides might fight bacterial cheating by controlling the expression and/or activity of bacterial enzymes that would divert the carbon flow of plant origin from energy production for nitrogen fixation to synthesize storage products such as PHB.

The biggest challenge concerning the function of nodule-specific peptides is the identification of their molecular targets and the effectors that mediate the bacterial responses. Their obvious targets are the bacterial membranes. It is known that cationic antimicrobial peptides (AMPs) can interact with the negatively charged surface of bacteria, which leads to pore formation or loss of the membrane integrity, culminating in lysis of the bacterial cells. Cationic NCRs can also interact with bacterial membranes and increase the membrane permeability (Van de Velde et al., 2010). Nevertheless, in the nodule cells the endosymbionts remain alive and cell lysis occurs only in the senescence zone. One possibility for the physiological role

of the membrane modifications is to enhance the nutrient exchange between the bacteroids and the peribacteroid space as well as the plant cells. Another option is that cationic peptides, by forming transient pores in the membranes, help the anionic peptides to enter bacterial cells and reach their intracellular targets. Cationic and anionic peptides are produced simultaneously in the same host cell and anionic peptides may reduce and control the cell-damaging activity of cationic peptides.

In addition, a fine balance between the antibacterial activity of cationic NCRs and resistance of rhizobia against the peptides is needed for the survival and differentiation of bacteria during nodule development. The BacA protein (discussed in detail in Chapter 31 by Guefrachi et al.) might contribute to this balance. BacA is essential for terminal bacteroid differentiation because *bacA*-deficient bacteria are more sensitive than the wild-type strains toward cationic NCRs *in vitro*, and they cannot survive in the NCR-loaded environment of symbiotic nodule cells (Haag et al., 2011). To identify intracellular targets of the individual nodule-specific peptides, genomic tools such as mutant and two-hybrid libraries in symbiotic partners, comparative transcriptome studies, as well as approaches using up-to-date techniques of cell biology and biochemistry are currently deployed.

What can be the evolutionary advantage for the plants to impose terminal differentiation onto the bacterial partner with the help of NCR peptides in the IRLC legumes? Oono and Dennison (2010) showed that terminally differentiated forms of bacteria outperformed reversibly differentiated bacteroids of the same strains. Why may rhizobia maintain a symbiotic lifestyle despite plant dominance? Nitrogen-fixing, terminally differentiated bacteroids have no reproductive capacity in nodules produced by IRLC legumes, and thus cannot pass their genes to the next generation and can be considered as a dead end of the lineage. However, the rhizobial population of a nodule is composed of the descendants of the bacterium that initiated the formation of the nodule including a subpopulation of undifferentiated rhizobia similar to free-living cells (Timmers et al., 2000). Inter- and intracellular presence of these rhizobia in the most proximal part of older *Medicago* nodules containing a senescence zone is the result of a non-controlled reinvasion process of the senescing nodule tissues by rhizobia from ITs. This colonization of the senescent cells leads to the differentiation of a post-senescence zone called saprophytic zone (or zone V). Rhizobia in zone V do not reduce nitrogen, but after nodule cessation they can escape into the soil and increase the bacterial population in the rhizosphere around the nodulated legumes and ensure the passing of the founder symbiotic bacterium's genes to the next generations.

67.6 ANTIMICROBIAL PEPTIDES IN OTHER SYMBIOTIC SYSTEMS

Maintaining a large bacterial population with host tissues and cells is not unique to legume nodules. For example, the gut of animals is crowded with symbiotic or commensal bacteria. The human gut has an estimated load of 10–100 trillion bacteria (10^{13}–10^{14} or 10–100 g of bacteria or the equivalent of 2–20 l of a saturated *Escherichia coli* culture!) representing several hundreds to thousands of species (Turnbaugh et al., 2007). Nevertheless, the human gut microbiota has a very specific species composition, suggesting an active selection mechanism by the host (Ley et al., 2006). The complexity of the gut microbiota can be very different among animals, and the bacterial diversity in the studied invertebrates is several orders of magnitude lower than in the mammals. Nevertheless, also in these cases the gut bacterial population has a very characteristic composition. For example, the *Drosophila* gut is dominated by just a few dominant bacterial species (Ryu et al., 2008). Even in *Cnidaria*, the simplest animals positioned in the earliest branches of the animal tree of life, the epithelial cells actively shape their bacterial gut community to a specific population structure (Fraune and Bosch, 2007). The gut microbiota is acquired from the environment and its assembly begins at the birth of the individual. It starts with a relative low level of bacterial diversity and gradually increases its complexity and changes its composition during the growth of the host (Fraune et al., 2010; Koenig et al., 2011).

Thus, the animal host has to build up its specific gut microbiota. Moreover, the gut bacteria are numerous and they may pose a threat to their host, requiring that the microbiota is maintained in a homeostatic relationship with the epithelial cells that are in contact with it. In mammalians, the innate and adaptive immune systems are among the host factors that determine the composition of the microbiota and keep them in equilibrium (Bevins and Salzman, 2011). Among many factors, AMPs of the innate immune system, produced by the epithelial cells and in particular by the Paneth cells, located in the intestine crypts, are primordial in the determination of the makeup of commensal microbiota. In transgenic mice with either reduced or increased production of defensins by the Paneth cells, the species composition of the microbiota is drastically changed but the total bacterial load remains unchanged and increasing or reducing defensin production has an opposing outcome (Salzman et al., 2010).

Based on very similar experimental strategies – manipulating the production of AMPs by epithelial cells of the intestine – these peptides were found to be key factors that shape the structure of the gut microbial community also in *Drosophila melanogaster* and in *Hydra* belonging to the Cnidaria (Ryu et al., 2008; Fraune et al., 2010). Thus,

host–produced AMPs are conserved metazoan key actors in the interaction with epithelial microbiota.

Examples of symbiotic interactions with a unique, specific bacterial species, hosted in large amount, also exist in the animal world. Many insects require bacterial symbionts producing nutrients that are missing in their diet. Notable examples are phytophagous insects that feed on plant sap or seeds. In most cases, the insect symbionts are housed intracellularly, in symbiotic organs called bacteriomes. Bacteriomes, the insect equivalent of the legume nodule, are constituted by bacteriocytes, the bacteriome equivalent of the nodule plant symbiotic cells.

The pea aphid *Acyrthosiphon pisum* is one of the best studied insects with respect to the bacterial symbiosis. *A. pisum* is in fact a species complex consisting of multiple biotypes, each of which is adapted to specific legume plants (e.g., pea, clover or *Medicago*). The primary [obligate] symbiont of *A. pisum* is *Buchnera aphidicola,* which produces essential amino acids lacking in the plant sap diet of the aphid. In addition, *A. pisum* biotypes may or may not have secondary or facultative symbionts belonging to a variety of bacterial lineages (e.g., *Hamiltonella*, *Serratia*, *Regiella*, *Rickettsia,* and *Spiroplasma*) and having diverse effects on the host (Oliver et al., 2010). Importantly, the bacterial symbionts in aphids and most insects are vertically transmitted, from mother to offspring, although facultative symbionts may undergo some horizontal transmission [acquisition from the environment]. Thus, the acquisition of the bacteria is a fundamental difference with the legume–*Rhizobium* symbiosis where the bacteria are exclusively acquired by horizontal transmission from the soil at each plant generation.

RNA-seq transcriptome analysis of bacteriocytes and *in situ* hybridization experiments in *A. pisum* have led to the discovery of a class of genes encoding secreted cysteine-rich peptides that were called BCR peptides for "bacteriocytes-specific cysteine rich peptides," in analogy to the NCR nodule peptides (Shigenobu and Stern, 2012). The genes are specifically expressed in the bacteriocytes and not in other parts of the insect. Following their expression by *in situ* hybridization during bacteriome formation in embryos, it was found that they are induced when the early-stage bacteriome becomes first infected with *Buchnera*. Thus, drawing the analogy with the NCR peptides in nodules it is an inevitable and exciting hypothesis that the BCRs are targeted to and affect the bacterial symbionts in a similar manner as the NCRs do in symbiotic nodule cells.

In a different insect group, in the stinkbugs or *Heteroptera*, symbiotic bacteria are not located in bacteriomes as intracellular endosymbionts but in a specialized, posterior region of the midgut where numerous crypts harbor in their lumen a single and specific extracellular bacterial symbiont (Kikuchi, 2009). The stinkbug *Riptortus pedestris,* which is another legume pest, particularly for soybean, carries in its crypts a symbiont belonging to the genus *Burkholderia*, a soil bacterium that is acquired every generation from the environment during a specific developmental window coinciding with the development of the crypts in the midgut (Kikuchi et al., 2011). Transcriptome analysis of symbiotic (crypt-carrying) and non-symbiotic midgut regions from *Burkholderia*-infected and uninfected (aposymbiotic) *R. pedestris* identified among the most abundantly expressed genes in the midgut 97 different cysteine-rich secretory peptides, many of which were specifically expressed in the *Burkholderia*-containing crypts of the midgut (Futahashi et al., 2013). The authors speculated that these crypt-specific cysteine-rich peptides are secreted into the lumen of the crypts by the epithelial cells and act on the proliferation and physiology of the symbiotic *Burkholderia*, similarly to the NCRs in legume nodules and the BCRs in aphid bacteriomes (Futahashi et al., 2013).

In yet another phytophagous insect, the weevil *Sitophilus*, which is a pest of cereals, bacteriomes carry the intracellular γ-Proteobacterium "*Sitophilus* primary endosymbiont" (SPE). SPE in bacteriocytes are large polyploidy and elongated bacteria reminding of the E-morphotype bacteroids in legume nodules. Bacteriocytes of *Sitophilus zeamais* produce a peptide, called coleoptericin-A (ColA), which is targeted to the endosymbionts (Login et al., 2011). ColA is unrelated to the legume NCRs, aphid BCRs, or stinkbug crypt-specific peptides and has no cysteine residues. The pure peptide induced very strong cell elongation with high DNA copy numbers on SPE. Downregulating the expression of the *colA* gene by RNAi resulted in decreased size and DNA content of the SPE in the bacteriomes and also in the spreading of the bacteria throughout the insect tissues. Thus, the role of the ColA peptide is to control the SPE endosymbiont, very analogous to the function of the NCRs in legume nodules.

These recent studies on insect symbiotic systems and animal gut microbiota, together with our findings in the legume–Rhizobium symbiosis, indicate that the employment of antimicrobial-like peptides in the control of symbiotic bacteria is widespread in nature and may be an optimal mechanism for eukaryotic host cells to maintain a homeostatic interaction with these bacteria.

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Chapter 68

The Symbiosome Membrane

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68.1 SYMBIOSOME FORMATION AND DEVELOPMENT

Rhizobia enter legume roots via infection threads that are initiated when the rhizobia are entrapped by curling of the root hair (Brewin, 2004). The local cell wall is then hydrolyzed and degraded, while the plasma membrane invaginates leading to the formation of a tubular infection thread. Infection threads then grow onward into the root cortex, transporting bacteria into the nodule primordium cells that are formed from reprogrammed root cortical cells (Brewin, 2004; Rae et al., 1992). Ultimately, the bacteria are internalized into nodule primordium cells and enter the plant cell cytoplasm surrounded by a host membrane, forming an organelle-like structure called the symbiosome (Fig. 68.1).

Roth et al. (1988) proposed the term "symbiosome" as a unified terminology to describe membrane-limited compartments that form as a result of endosymbiotic interactions. They defined the symbiosome as a "membrane-bound compartment containing one or more symbionts and certain metabolic components and located in the cytoplasm of eukaryotic cells" (Roth et al., 1988). The membrane around this structure can be termed the "symbiosome membrane" (SM). We use this term here instead of the more common term of peribacteroid membrane, which ignores the general nature of such structures (Roth et al., 1988). It is important to appreciate that the SM and the bacteroid membrane (BM) are physically separated by a symbiosome space, and therefore their transport activities are distinct. Since the bacteroid is enveloped by the SM as a result of an endocytosis-like process of the plasma membrane, the SM is inverted compared to the plasma membrane, and hence metabolite movement from the infected cell cytosol to the symbiosome space (across the SM) is actually exported from the plant cell; conversely, efflux of metabolites from the symbiosome represents uptake into the plant cytoplasm (White et al., 2007). Symbiosomes undergo four developmental phases, namely initiation, proliferation, maturity, and degradation. The role and composition of the SM is considered to be different in the four phases (Brewin, 1991).

Symbiosome biogenesis begins with the formation of an infection droplet, a structure that represents an unwalled outgrowth from the infection thread. This is a region where the infection thread membrane invaginates and where rhizobia come into direct contact with the host plasma membrane without the cell wall as a barrier (Brewin, 2004; Rae et al., 1992). Bacteria are then released into the cytoplasm of cortical cells, which results in encapsulation of bacteria by the plant cell membrane (Bassett et al., 1977; Goodchild and Bergersen, 1966; Newcomb, 1976). Inside the symbiosome, bacteria differentiate into bacteroids, their symbiotic form, which enables nitrogen fixation to take place

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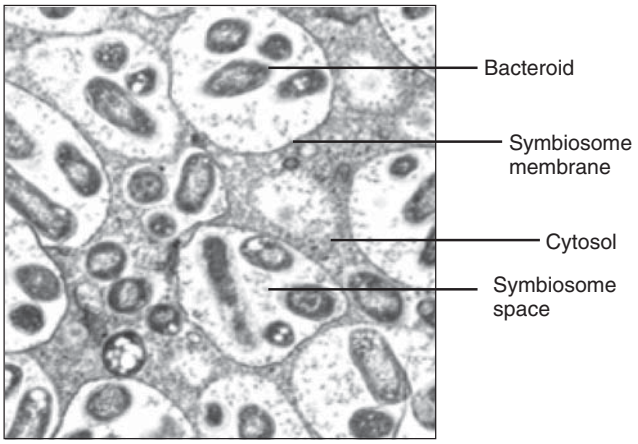

Figure 68.1 Electron micrograph of symbiosomes within infected cell of a soybean nodule.

(Vasse et al., 1990). In indeterminate nodules of temperate legumes (e.g., alfalfa, pea), symbiosomes typically house a single large bacteroid; in determinate nodules of tropical legumes (e.g., soybean), mature symbiosomes fuse to form large structures that house many bacteroids (Fedorova et al., 1999). Formation of symbiosomes involves a major reorganization of host cytoskeleton and endomembrane systems (Davidson and Newcomb, 2001). To accommodate biogenesis of symbiosomes in the infected cells, massive amounts of protein and lipid are needed. Depending on the legume species, the total surface area of SM can exceed the plasma membrane by approximately 30-fold (Verma et al., 1978). Although traditionally it is considered that the symbiosome forms through endocytosis, more recent evidence suggests that the exocytotic pathway also plays a major role.

In endocytosis, internalized cargo molecules that are taken up by the plasma membrane are transported through the endocytic pathway (Šamaj et al., 2005). The endocytotic pathway involves a multifaceted network of membrane compartments, with each compartment involved in different tasks. After internalization into the early endosome, cargo to be recycled is transported back to the plasma membrane, whereas cargo for degradation is transported to the late endosome. The late endosome will finally fuse with the vacuole in plants or lysosomes in animals for degradation (Šamaj et al., 2005).

Limpens et al. (2009) showed that in *Medicago truncatula*, symbiosomes did not acquire the (late) endosomal marker Rab5 or early endosomal/trans-Golgi network (TGN) marker, SYP4, at any stage during their development and only acquired Rab7, a late endosomal/vacuolar marker, at a later stage of development when symbiosomes had stopped dividing (the fixation zone). This marker was retained until senescence began. This suggests that the early formation of the symbiosome follows a Rab5-independent endocytic pathway, and that Rab7 might be recruited directly from the cytoplasm to regulate symbiosome formation. In *M. truncatula*, symbiosomes did not acquire vacuolar SNARE, SYP22, and VTI11 until the onset of senescence. However, the SM contains a plasma membrane t-SNARE/SYNTAXIN SYP132 throughout development (Catalano et al., 2007; Limpens et al., 2009). The delay in acquiring vacuolar SNAREs might be required for maintenance and survival of the symbiosome as an individual nitrogen-fixing organelle. The presence of SYP132 and Rab7 showed that the SM has a unique composition where proteins that move from the secretory pathway to the plasma membrane and from the endocytic pathway to the vacuole are involved (Limpens et al., 2009). The involvement of Rab1 and Rab7 GTPase in SM formation has been demonstrated in soybean (Cheon Iii et al., 1993). Nodules expressing an antisense construct for Rab1 were smaller and had fewer bacteroids per cell, and the vacuole was retained after bacteria were released into the cytoplasm. *In situ* hybridization with a soybean Rab7 probe on nodules at different development stages showed that the transcript was present in the infected zone early in the development of the nodule (7 days after infection) (Son et al., 2003). There is no clear molecular evidence for involvement of the default endocytosis pathway in the early stages of symbiosome formation.

In contrast, evidence of the involvement of an exocytotic pathway is accumulating (Ivanov et al., 2012). Exocytosis involves fusion of transport vesicles with the plasma membrane. This fusion is mediated by exocytic vesicle-associated membrane proteins (VAMPs) and in plants, which are members of the VAMP72 family. Silencing of two *VAMP72* family members in *M. truncatula* prevented symbiosome formation, supporting the role of exocytosis in their formation. GFP-VAMP72 fusions localized to dot-like structures where unwalled infection droplets are formed and symbiosomes start to develop. The unwalled infection droplets are surrounded by host membrane and cell wall is completely absent. Bacteria are subsequently pinched off when they come into close contact with the host membrane. In the VAMP72 silenced nodules, infection droplets did not form properly as they were bound within a thin layer of cell wall. The presence of the cell wall prevents close contact between bacteria and host membrane, which prevents further release of bacteria (Ivanov et al., 2012). The involvement of DNF1 (defective in nitrogen fixation), which encodes a signal peptidase complex involved in a nodule-specific secretory pathway, is further evidence that supports the formation of the symbiosome via exocytosis (Wang et al., 2010).

Bergersen and Briggs (1958) were the first to observe the SM, which consists of a membrane bilayer of 9–10 nm thick (Dart and Mercer, 1963; Mellor and Werner, 1987). The SM is derived from the plasma membrane through the infection thread membrane, as illustrated by staining by phosphotungstic acid-chromic acid (PACA) (Robertson et al., 1978a;

Roth and Stacey, 1989; Verma et al., 1978), which is usually considered a plasma membrane-specific stain. The syntaxin SYP132, as well as being present in the SM, labels the plasma membrane around the infection threads and infection droplets (Catalano et al., 2007), also linking the two membranes.

The SM proliferates and differentiates to accommodate bacteroid growth and division until the infected cells are filled with symbiosomes (Robertson and Lyttleton, 1984). Enlarged mature infected cells may house thousands of symbiosomes (Day et al., 2001). It is still unclear how the SM proliferates, but it is proposed that it may be the result of redirection of the host secretory pathway toward SM biogenesis (Leborgne-Castel et al., 2010). Proliferation of the SM is considered to occur independently of bacteroid division (Mellor and Werner, 1987). Within the developing infected cell, there is a specialized area adjacent to the nucleus that is devoid of symbiosomes where abundant membrane is observed associated with the endoplasmic reticulum (ER). This was suggested to serve as a membrane reservoir for the proliferating symbiosomes (Bulbul and Kaneko, 2009). Bundles of actin filaments that colocalized at the same site may function in delivering membranes to the developing symbiosomes (Bulbul and Kaneko, 2009) and play a role in spatial organization of the symbiosomes, maintaining effective pathways for nutrient diffusion within the symbiosomes (Whitehead et al., 1998a).

Notwithstanding the origin of the symbiosome, bacteroid division and SM proliferation result in an SM with distinct properties. Both the protein and lipid complement of the SM are unique and are supplied from a range of sources, including the ER and *de novo* membrane synthesis (Roth and Stacey, 1989). Galactolipid digalactosyldiacylglycerol (DGDG) lipids, which are usually associated with plastid membranes rather than plasma membrane, are present in the SM (Gaude et al., 2004), indicating its unique identity. The mature SM also has an exceptionally high lipid-to-protein ratio (6:1) (Robertson et al., 1978a). The phospholipid and fatty acid contents of the SM most closely resemble that of the endomembrane system (the ER and Golgi apparatus) (Bassarab et al., 1989; Mellor et al., 1985) rather than the plasma membrane. The SM also contains a high level of phosphatidylcholine, but low levels of phosphatidylethanolamine compared to the plasma membrane (Hernández and Cooke, 1996). The SM contains β-amyrin, a novel plant triterpenoid that is not accumulated in the root plasma membrane (Bassarab et al., 1989; Hernández and Cooke, 1996).

ER and Golgi apparatus are observed close to symbiosomes (Kijne and Pluvqué, 1979; Whitehead and Day, 1997), suggesting that they are involved in transport of newly synthesized proteins and lipids to the SM via vesicles from the Golgi, but there is little information available about how proteins are targeted to the SM. Targeting of two symbiosome space proteins has been investigated. For NOD25 from *M. truncatula*, a 24 amino acid signal peptide (SP) was sufficient to target green fluorescent protein (GFP) to the symbiosome space across the SM (Hohnjec et al., 2009). This suggests a posttranslational pathway for its import. The peptide was conserved in two other proteins known to be targeted to the symbiosome. Similarly, the early nodulin 8 (MtENOD8) SP could direct GFP across the SM but two other domains in the protein also had this ability, suggesting redundancy in the targeting signals (Hohnjec et al., 2009; Meckfessel et al., 2012). Further analysis of other symbiosome space and SM proteins is required to determine whether N-terminal SPs are a common feature and whether different proteins follow a common pathway to reach these destinations.

In indeterminant nodules such as *M. truncatula* and *Pisum sativum*, the meristemic activity of the apical meristem results in the formation of a gradient of developmental stages that are defined as four zones: the meristematic zone at the distal end, providing new cells to the nodules; the infection zone, where bacteria are released into the cytoplasm, forming the symbiosome; the fixation zone, where symbiosomes are completely differentiated and the *nif* genes, encoding the rhizobial nitrogenase complex, are induced (de Maagd et al., 1994); and the senescence zone at the proximal end where symbiosomes degrade (Gage, 2004). Determinate nodules such as *Glycine max* and *Lotus japonicus* lack a persistent meristem (Brewin, 1991) and have a central infected zone surrounded by nodule parenchyma. In both the fixation zone of indeterminate nodules and the infected zone of determinant nodules, large infected cells are interspersed with smaller uninfected cells. The uninfected cells are thought to accumulate reduced carbon such as sucrose and convert these to organic acids, which are then transported to the infected cells through symplastic transport (Peiter et al., 2004; White et al., 2007). In determinant nodules, they are also important in assimilation of fixed nitrogen and are the site of ureide synthesis, which in most determinant nodules is the form in which nitrogen is transported to the shoot (Smith and Atkins, 2002). Senescence of symbiosomes usually begins at week 5 after inoculation (Vasse et al., 1990). The symbiosomes are targeted and fused to the lytic vacuole for degradation.

68.2 TRANSPORT ACTIVITY OF THE SYMBIOSOME MEMBRANE

A key role of the SM is to mediate and regulate the exchange of nutrients and metabolites between the symbiotic partners in a way that optimizes nitrogen fixation (Brewin, 1996). The SM may also play a role in protecting the bacteria from plant defense mechanism (Mellor and Werner, 1987; Vance, 1983) and plants from any pathogenic behavior from the rhizobia (Brewin, 1996).

Information on the transport activity of the SM is essential to determine what regulates the amount of nitrogen fixed and how bacteroids retain a symbiotic relationship with the host plant (Rosendahl et al., 1991). Our knowledge of the transport activity of the SM mostly results from biochemical studies and transport assays using isolated symbiosomes. Transport assays using radiolabeled substrates and patch-clamp techniques have provided insights into the nature of the metabolites that cross the SM, but the molecular identities of most of the transporters involved remain largely unknown. With the availability of complete genome sequences of several legumes (http://phytozome.net/soybean: (Schmutz et al., 2010); http://www.kazusa.or.jp/lotus: (Sato et al., 2008); http://medicago.org/genome: (Young et al., 2011)), molecular characterization of these transporters is now possible.

To support nitrogen fixation, the plant must provide a carbon source to the bacteroid in exchange for fixed nitrogen. Reduced carbon for fixed nitrogen is the principal metabolite exchange between the symbiotic partners, but the bacteroid is dependent on the plant for many other (micro)nutrients that include iron, molybdenum, vanadium, nickel cobalt, sulfur, selenium, phosphate, and homocitrate (Hakoyama et al., 2009; Rosendahl et al., 1991).

68.2.1 Carbon Supply to the Bacteroid

Carbohydrate breakdown in nodules provides substrates for bacteroid metabolism and carbon skeletons for the assimilation of fixed ammonia into organic acids. The principal carbon and energy supply for nodule metabolism is derived from recently fixed plant photosynthetic carbon compounds in the form of sucrose. There is a large body of evidence that bacteroids derive their energy from oxidative respiration of organic acids, principally malate (Day, 1991 and references therein; Fig. 68.2). Bacteroids that are mutant for the dicarboxylate transporter encoded by *dctA* are unable to fix nitrogen (reviewed in Yurgel and Kahn, 2004). Isolated symbiosomes are able to take up dicarboxylic acids (but not sucrose) at rapid rates, and a transporter with specificity for univalent malate anions has been identified on the SM of soybean (Udvardi, 1988) and French bean (Herrada et al., 1989). However, the gene(s) encoding the dicarboxylate transporter remains to be identified. In nodules of actinorhizal Alder plants, a cDNA, *AgDCAT1*, was identified, and the protein it encoded was localized to the symbiotic interface (Jeong et al., 2004). AgDCAT1 is a member of the proton-dependent oligopeptide transporter (POT) family, and it was able to transport dicarboxyclic acids including malate, succinate, fumarate, and oxaloacetate when expressed in *E. coli*. Members of the same family are strongly induced during nodule development in *G. max* (Libault et al., 2010; Severin et al., 2010), *M. truncatula* (Benedito et al., 2008), and *L. japonicus* (Colebatch et al., 2004). However, members of the POT family are known to transport a range of different compounds including nitrate, peptides, auxin, and glucosinolates (Saier and Milton, 2000), so further functional characterization will be required to determine if those upregulated in nodules encode a malate transporter.

There is a range of protein families that include malate transporters in plants, and it is possible that a member of one of these could be the SM malate transporter. Members of the aluminum-activated malate transporter (ALMT) family promote organic acid efflux from the roots of nonlegumes (Sasaki et al., 2004) and regulate cytosolic malate homeostasis when localized on the vacuolar membrane (Kovermann et al., 2007). The divalent anion/Na^+ symporter (DASS) family (also called SLC13 family) mediates the transport of dicarboxylates and inorganic anions across the cellular membrane in a Na^+ -dependent manner (Hall and Pajor, 2005) and includes the *At*tDT dicarboxylate transporter from *Arabidopsis thaliana*, which is targeted to the vacuolar membrane in leaf cells (Emmerlich et al., 2003). The ATP-binding cassette (ABC) transporter AtABCB14 transports malate from the apoplast into the guard cells in *Arabidopsis* to modulate responses to CO_2 (Lee et al., 2008). As an importer of malate, the direction of AtABCB14 transport is not what would be considered for the SM malate transporter, which is expected to be related to the families that efflux malate. Although these families have members in legumes, there is little indication that any family has enhanced expression in nodules.

68.2.2 Fixed Nitrogen

Ammonia, NH_3, or ammonium ions, NH_4^+, are believed to be the most likely in the form of fixed nitrogen provided to the plant (Day et al., 2001; Udvardi and Poole, 2013). NH_3 is the product of the nitrogenase enzyme in the bacteroid and is transported out of the bacteroid into the symbiosome space, probably by simple diffusion. Enzymes involved in the ammonium assimilation pathway [glutamine synthetase (GS)/glutamine oxoglutarate amidotransferase (GOGAT)] are repressed in the bacteroid, while those in the plant are elevated, thus creating a driving force down the concentration gradient for the efflux of NH_3 out of the bacteroid (Atkins, 1991; Poole and Allaway, 2000). The symbiosome space is more acidic than the bacteroid space, and thus NH_3 that leaves the bacteroid is protonated, forming NH_4^+ ions. It was initially proposed that the transport of NH_3/NH_4^+ from the symbiosome space to the plant cytosol occurs via diffusion (Udvardi and Day, 1990). Later studies used patch-clamp techniques on isolated symbiosomes to identify a monovalent cation channel capable of NH_4^+ transport across the SM in soybean (Tyerman et al., 1995), pea (Mouritzen and Rosendahl, 1997) and *L. japonicus* (Roberts

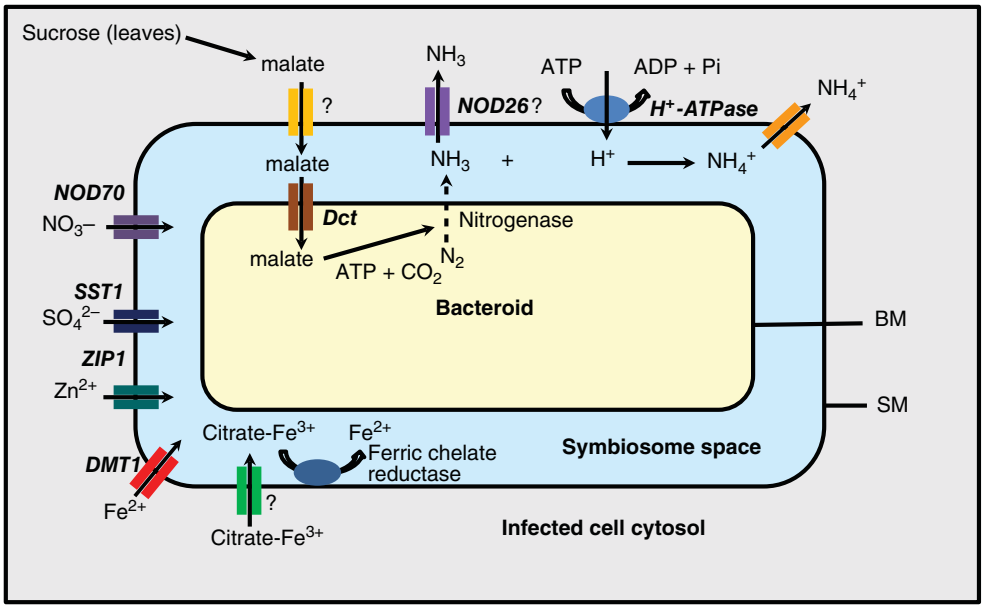

Figure 68.2 Diagrammatic representation of transport processes in the symbiosome of infected cell. Sucrose derived from photosynthesis is the major reduced carbon supply for nodule metabolism. Dicarboxylate acids (principally malate) are supplied to the bacteroids for oxidative respiration and nitrogen fixation. The gene encoding the dicarboxylate transporter on the SM has not been identified. On the BM, a dicarboxylate transport (Dct) system is responsible for importing dicarboxylates into the bacteroids. The product of nitrogenase enzyme, NH_3, is transported out of the bacteroid, probably by simple diffusion. NH_3 is either protonated into NH_4^+ and leaves the symbiosome via a monovalent cation channel or remains unprotonated and leaves the symbiosome via NH_3 channel proposed to be Nodulin 26. Fe is supplied to the symbiosome as either Fe(II) via DMT1 or Fe(III)-citrate via an uncharacterized Fe(III) transporter that will then be chelated by ferric chelate reductase, which is located on the SM side. Sulfate, SO_4^{2-}, is transported into the symbiosome via SST1 and zinc, Zn^{2+} via ZIP1. An anion transporter with a preference for nitrate, NO_3^- is encoded by NOD70.

and Tyerman, 2002; Fig. 68.2). These channels open when membrane potential is generated through acidification of the symbiosome space by a proton pumping H^+-ATPase on the SM (Blumwald, 1985) and outward rectification of the channel by the presence of Mg^{2+} on the cytosolic side of the membrane (Whitehead et al., 1998b). As a consequence, movement of NH_4^+ is unidirectional – out of the SM, and thus there is no backflow of NH_4^+ into the symbiosome. However, there is also evidence that some NH_3 may diffuse across the SM through a channel (Niemietz and Tyerman, 2000), proposed to be Nodulin 26.

68.2.3 Nodulin 26

Nodulin 26 is a major component of the SM, which makes up 10% of the total SM protein (Rivers et al., 1997) and is a member of the major intrinsic protein (MIP), membrane protein channel superfamily that includes aquaporin water transporters (see Chapter 69). Nodulin 26 may transport NH_3 from the symbiosome (Niemietz and Tyerman, 2000; Fig. 68.2), and its C-terminal domain was recently shown to interact with cytosolic GS (Masalkar et al., 2010). It is proposed that the interaction with GS creates a metabolic channel to aid assimilation of fixed nitrogen and prevent futile cycling of ammonia (Masalkar et al., 2010). It is interesting to note that other members of the superfamily, such as *Arabidopsis* tonoplast intrinsic protein (TIP) family, and mammalian AQP1 and AQP8 also transport ammonia (Jahn et al., 2004; Loqué et al., 2005; Zeuthen et al., 2009). Earlier studies on Nodulin 26 suggested that Nodulin 26 has high permeability to water and also transports glycerols and formamide (Dean et al., 1999; Guenther et al., 2003; Rivers et al., 1997), and that its permeability may be regulated by phosphorylation (Weaver and Roberts, 1992). The water transport activity of Nodulin 26 may be important for osmoregulation during the symbiosis (Dean et al., 1999; Guenther et al., 2003). Nodulin 26 reconstitution into proteoliposomes results in NH_3 transport and has approximately fourfold stronger preference for NH_3 over water (Hwang et al., 2010).

68.2.4 Energization of the Symbiosome Membrane

The SM is energized by a H^+-pumping ATPase that resembles a P-type plasma membrane ATPase (Fedorova et al., 1999; Whitehead and Day, 1997) and facilitates the exchange of malate and ammonium across the SM (Udvardi and Day, 1997; Fig. 68.2). ATPase activity was one of the first enzyme activities to be recorded on the SM (Robertson et al., 1978b; Verma et al., 1978). Biochemical studies on soybean

(Bassarab et al., 1986; Blumwald, 1985; Udvardi, 1989), lupin (Andreev et al., 1997; Domigan et al., 1988), and pea (Szafran and Haaker, 1995) suggest that ATPase activity on the SM is Mg^{2+} dependent, possesses slightly more acidic pH optimum than the plasma membrane-type ATPase, is inhibited by vanadate, and is stimulated by cations especially K^+ and NH_4^+. ATPases generate a pH gradient across the SM by pumping protons into the symbiosome space while the bacteroid respiratory electron transport chain pumps protons out of the bacteroid into the symbiosome space. Consequently, the symbiosome space is acidified. This creates SM electrochemical gradients, which drive exchange of malate and ammonium across the SM (Udvardi and Day, 1997).

68.2.5 Iron

Iron supply for nitrogen fixation is important, as it is required for synthesis of various iron-containing proteins in both bacteroids and plants. In bacteroids, iron is required for nitrogenase synthesis and cytochromes that are used in the bacteroid electron transport chain, while in the plant, iron is a constituent of the heme moiety of leghemoglobin, which facilitates the diffusion of oxygen to the symbiosomes (Appleby, 1984). Iron is transported across the SM either in the form of Fe(II) by an Fe(II) transporter/channel (Moreau et al., 1998) or in the form of Fe(III)-chelate (LeVier et al., 1996; Moreau et al., 1995; Fig. 68.2). The assays used to show iron transport measured radioactive iron uptake into isolated symbiosomes. Most of the Fe(III) taken up by symbiosomes appeared to accumulate in the symbiosome space, but not in the bacteroids as these do not take up ferric iron readily (LeVier et al., 1996). Fe(III) transported across the SM to the symbiosome space may be bound by siderophore-like compounds released by bacteroids, helping to retain iron in the symbiosome space (Wittenberg et al., 1996). This has led to speculation that the symbiosome space may be the major storage of Fe in the nodule (Udvardi and Day, 1997). However, the identity of the Fe(III) transporter has not been determined.

Fe(II) uptake across the SM in soybean has been suggested to be mediated by GmDMT1 (Kaiser et al., 2003), a homolog of the natural resistance-associated macrophage protein (NRAMP)/Dmt1 family of divalent metal transporters. This family transport mainly divalent metal irons including Fe^{2+}, Mn^{2+}, and Cd^{2+} (Cellier, 2012). GmDMT1 was localized on the SM of infected cells and was able to complement a yeast strain deficient in Fe(II) transport (Kaiser et al., 2003). The proposed mechanism of iron transport via DMT1 raises an interesting problem regarding the orientation of DMT1 in yeast. Its localization in yeast was not confirmed, but was suggested to be on the plasma membrane due to its ability to take up iron from the media. Uptake of Fe(II) into the cell cytoplasm is equivalent to transport out of the SM, although clearly it can be taken up into the symbiosome. This suggests that DMT1 may catalyze bidirectional transport of iron (Kaiser et al., 2003). The importance of DMT1 for nitrogen fixation *in planta* has not been assessed. The integral membrane protein, SEN1 from *L. japonicus* that is related to the yeast CCC1 iron/manganese transporter and the *Arabidopsis* VIT1 vacuolar iron transporter, is essential for nitrogen fixation (Hakoyama et al., 2012). It is postulated that SEN1 transports iron across the SM although its localization in infected cells has not been determined experimentally. Its expression is specific to infected cells in the nodule, and symbiosome development and bacteroid differentiation are impaired in the *sen1* mutant nodules (Hakoyama et al., 2012).

68.2.6 Zinc

Transport of zinc across the SM is mediated by GmZIP1 in soybean (Moreau et al., 2002; Fig. 68.2). GmZIP1 is a member of the ZRT [zinc-regulated transporter], IRT [iron-regulated transporter]-like protein (ZIP) family, which transports a wide range of divalent cations that include Cd^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+} (Guerinot, 2000; Lin et al., 2009; Pedas et al., 2008). An anti-GmZIP1 antibody-inhibited uptake of radiolabeled zinc into isolated symbiosomes (Moreau et al., 2002) and ZIP1 was able to complement a zinc transport deficient yeast strain. As for DMT1, the orientation of ZIP1 in yeast suggests that it may be able to mediate bidirectional transport of zinc. Although it is unclear whether GmZIP1 is vital for the symbiosis, *GmZIP1* mRNA is expressed in nodules after the onset of nitrogen fixation (in plants 18 days after inoculation with rhizobia), suggesting its importance in zinc metabolism in the nodules. Zinc plays a key role in the regulation of genes involved in phosphorus uptake in plants (Huang et al., 2000; Webb and Loneragan, 1988), and it has been postulated that zinc may play a role in regulating phosphorus levels in nodules (Moreau et al., 2002).

68.2.7 Nitrate

Nitrogen-fixing symbioses are induced in nitrate-deficient soil, and nitrate is believed to be the preferred form of nitrogen for the plant. Thus, in the presence of nitrate, nodule development is suppressed, nitrogen fixation is inhibited, and senescence is initiated (Caroll and Mathews, 1990; Drevon et al., 1988; Streeter and Wong, 1988). A putative transporter GmN70 has been cloned in soybean and localized to the SM of infected root cells using western blot and immunocytochemical analyses (Vincill et al., 2005; Fig. 68.2). GmN70 is an ortholog of the nodulin LjN70 of *L. japonicus*, a protein member of the major facilitator superfamily (MFS) that is also localized to the SM (Szczyglowski et al., 1998). GmN70 and LjN70 are proposed to be anion transporters with a preference for nitrate, which regulates ion and membrane potential homeostasis,

particularly in nitrate concentrations in the symbiosome space (Vincill et al., 2005). The high selectivity for nitrate is rather interesting as nitrate has been found to inhibit nitrogen fixation (Arrese-Igor et al., 1997). There are many proposed reasons as to why this might happen (reviewed in Luciński et al., 2002). One is because the presence of nitrate may increase oxygen diffusion resistance in the root cortex, which then caused a decline in bacteroid oxidative respiration (Minchin et al., 1986). Alternatively, increased concentrations of nitrate may lead to a reduction in membrane potential, across the SM and consequently inhibit transport of nutrient/metabolites across the SM (Udvardi, 1989).

68.2.8 Sulfate

The *Sst1* gene, which encodes a transporter thought to be specific for sulfate that is localized on the SM in *L. japonicus* (Wienkoop and Saalbach, 2003; Fig. 68.2), is essential for nitrogen fixation (Krusell et al., 2005). *Sst1* is highly induced during nodule development (Colebatch et al., 2002), and when expressed in yeast was able to complement a yeast mutant deficient in sulfate transport (Krusell et al., 2005). SST1 is proposed to transport sulfate from the plant cell cytoplasm to the symbiosome space. Sulfur is an essential component of the nitrogenase metalloenzyme, where each active nitrogenase complex consists of two NifH, a NifD and a NifK subunit, and four metal–sulfur clusters, which provide a medium for electron transfer to the N_2 (Dos Santos et al., 2004; see Chapters 2, 7). Synthesis of nitrogenase is impaired in *sst1* mutant plants. The role of SST1 and its importance to nitrogen fixation raises some questions as symbiosomes isolated from soybean do not take up sulfate (D Day unpublished observation). Some members of the symbiosome sulfate transport (SST) family are able to catalyze transport of other substances including molybdate (Tomatsu et al., 2007), which is also essential for nitrogenase and nitrogen fixation. Thus, it is possible that this transporter has a physiological role on the SM that does not include sulfate transport.

68.2.9 Amino Acids

There is evidence in pea that branched-chain amino acids, namely leucine, isoleucine, and valine (LIV), are required for effective nitrogen fixation, and that they must be provided by the plant across the SM (Prell et al., 2009). *Rhizobium leguminosarum* bv. *viciae* contains two broad specificity amino acid ABC transporters, AapJQMP and BraDEFGC (Hosie et al., 2002; Walshaw, 1996). When *aap bra* double mutants were inoculated on pea, *P. sativum*, the bacteroids appeared morphologically normal under electron micrographs, but the plants exhibited a nitrogen-starved phenotype and increased nodule number and mass (Lodwig et al., 2003). The plants only fixed nitrogen at around 30% of wild-type rates (Lodwig et al., 2003). By altering the specificity of Bra to only transport branched-chain amino acids of LIV, Prell et al. (2009) demonstrated that LIV transport is essential for effective nitrogen fixation in peas. The overexpression of glutamate dehyrogenase, *gdhA*, in bacteroids did not rescue the *aap bra* mutants, suggesting that the dependency of bacteroids on the plant for LIV is not because of the lack of ammonium incorporation into amino acids resulting from the known downregulation of glutamate synthesis. Instead, the effect is caused by transcriptional downregulation of LIV biosynthesis in bacteroids, which become dependent on amino acids supplied by plants (Prell et al., 2009). This event is called symbiotic auxotrophy. Studies with isolated soybean symbiosomes have failed to provide evidence for carrier-mediated uptake of amino acids, including branched-chain amino acids, but significant diffusion of these compounds across the SM was observed (Udvardi et al., 1990). However, since only a low rate of LIV transport is needed to overcome symbiotic auxotrophy in *R. leguminosarum* (Prell et al., 2010), diffusion across the SM may be sufficient to sustain the symbiosis.

68.2.10 Homocitrate

Homocitrate is a component of iron-molybdenum (FeMo) cofactor of nitrogenase complex (Hoover et al., 1989; see Chapter 2), and so homocitrate is essential for nitrogen fixation. Supply of homocitrate from the plant to the bacteroid may be necessary as most rhizobia, except in *Azorhizobium caulinodans* and *Bradyrhizobium* sp. lack a *NifV* gene encoding homocitrate synthase (HCS) (Hakoyama et al., 2009; Zheng et al., 1997). In *L. japonicus*, *FEN1* encodes HCS (Hakoyama et al., 2009) and *fen1* mutant nodules have very low nitrogenase activity and ineffective nodules (Imaizumi-Anraku et al., 1997). *Mesorhizobium loti* carrying *FEN1* is able to rescue the *fen1* mutant (Hakoyama et al., 2009). These results suggest that homocitrate must be transported across the SM, but to date no such transporter has been identified.

68.3 CONCLUSION AND FUTURE WORK

Given that the symbiosome is an integral part of the legume–*Rhizobium* symbiosis and the SM is a key to the interaction between the symbionts, it is surprising how little is understood about its development, protein composition, and functional role. The recent evidence for a role of exocytosis in symbiosome formation is exciting, but details are only beginning to appear and we know very little about targeting of proteins to the SM. Clearly, this is an area in need of intense study. Likewise, while early biochemical studies

provided a picture of what substances can be transported across the SM and in some instances pointed to the probable mechanisms involved, we still know very little about the molecular identity of the transport proteins involved, nor the way that the genes encoding these proteins are regulated. The recent legume genome sequencing projects and accompanying transcriptome analyses (see earlier) promise to accelerate a more detailed analysis of SM synthesis and transport properties. In particular, accurate proteomic analysis of the SM is now possible and is yielding valuable insights into novel transporters and other integral membrane proteins on this unique membrane.

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Section 12

Nitrogen Fixation, Assimilation, and Senescence in Nodules

Chapter 69

Nodulin Intrinsic Proteins: Facilitators of Water and Ammonia Transport across the Symbiosome Membrane

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69.1 INTRODUCTION

During the establishment of legume–rhizobia symbioses, rhizobia infect and occupy specialized infected cells within the core of the symbiotic root nodule. Nitrogen-fixing rhizobia bacteroids reside within nitrogen-fixing organelles called symbiosomes (Roth et al., 1988; see Chapter 68). In mature nodules, the host-infected cells are occupied by thousands of symbiosomes, which constitute the major organelle within this cell type. The plant-derived membrane "symbiosome membrane" (SM) encloses the endosymbiotic rhizobia bacteroid, and possesses transport and channel activities involved in the exchange of metabolites and signaling molecules between the host and symbiont that are essential for the support of symbiotic nitrogen fixation (reviewed in Day et al., 2001; White et al., 2007; Udvardi and Poole, 2013; see Chapter 68). These include the central metabolic exchange that characterizes the symbiosis: (i) the uptake of a carbon energy source, generally in the form dicarboxylates, provided by the plant and utilized by the bacteroid to produce adenosine triphosphate (ATP) in support of nitrogen fixation; and (b) the release of the reduced product of nitrogen fixation as either ammonia (NH_3) or ammonium ion (NH_4^+) (Fig. 69.1).

Apart from this fundamental carbon/nitrogen exchange, the SM possesses multiple transport functions to facilitate the uptake of various other metal cofactors, as well as other components needed to support the symbiosis (reviewed in Udvardi and Poole, 2013; see also Chapter 68). As a result, biogenesis of the symbiosome is accompanied by the expression of a number of transporter and channel proteins, many of which are targeted to the SM. Among these are a class of plant-specific aquaporin-like channel proteins known as "nodulin 26 intrinsic proteins" (NIPs) (Wallace et al., 2006). In the present chapter, we consider the potential multifunctional roles of NIP channels in metabolic and osmoregulation in nodules, as well as the potential interplay between these proteins, ion channels, and glutamine synthetase (GS) in mediating ammonia efflux and assimilation.

69.2 THE NIP SUBFAMILY: PLANT-SPECIFIC CHANNELS OF THE AQUAPORIN SUPERFAMILY

Nodulin 26 was originally discovered as the major protein component of the soybean SM (Fortin et al., 1987) and as a target for calcium-dependent protein kinases localized on this membrane (Weaver et al., 1991). It was noted that the protein possessed homology to proteins of the aquaporin superfamily of water and solute channels, and it was among the first members of this ancient channel superfamily discovered in higher plants (Sandal and Marcker, 1988). Biochemical and proteomic studies of other legume symbiosomes indicate that nodulin 26-like proteins are major protein components (Guenther and Roberts, 2000; El Yahyaoui et al.,

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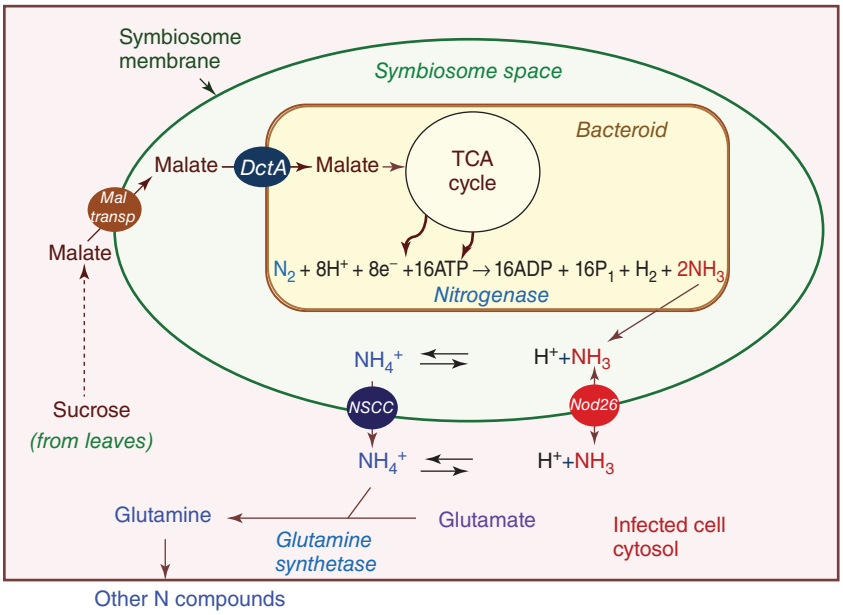


Figure 69.1 Carbon and nitrogen flux in nitrogen-fixing symbiosomes. Infected cells convert sucrose provided by the shoot to malate, which is transported to the bacteroid via SM and the bacteroid dicarboxylate transporters. Malate serves as the catabolic energy source that provides energy for nitrogenase-catalyzed fixation of N_2. Reduced ammonia is transported to the symbiosome space and is effluxed to the infected cell cytosol by one of two pathways (NSCC or nodulin 26, see text for details), where it is assimilated (see Chapter 68).

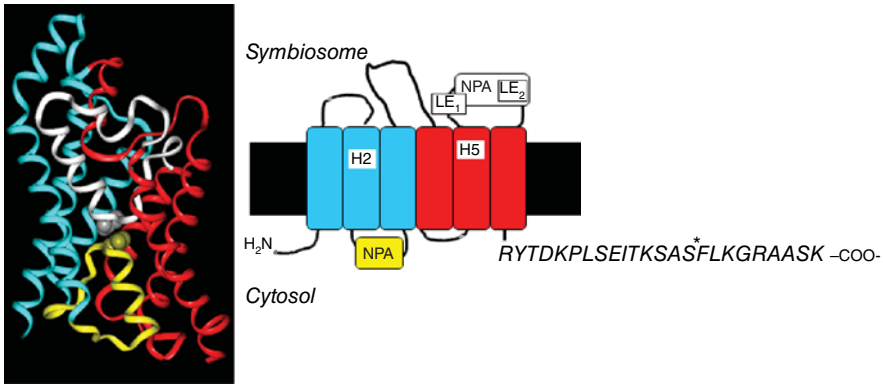


Figure 69.2 Schematic diagram of the topology of nodulin 26. (Right) The nodulin 26 topology based on structural modeling (Wallace and Roberts, 2004) is shown. The topology model shows the typical six transmembrane α-helices and twofold symmetry characteristic of the aquaporin superfamily. The two small helices that contain the conserved NPA motifs are shown in white and yellow. H2, H5, LE_1, and LE_2 indicate the four amino acids that form the selectivity filter in the channel pore. The asterisk shows the position of the CDPK phosphorylation site. (Left) A backbone ribbon structure illustrating the conserved hourglass fold of the aquaporin superfamily is shown based on the X-ray crystal structure of aquaporin 1 (Sui et al., 2001). The structure shows the disposition of the various helices and NPA motifs with the same color scheme shown in the topology model.

2004; Catalano et al., 2004), and thus are likely to play a common and integral role in transport/channel processes in the SM of most if not all legume/rhizobia symbioses.

As a member of the aquaporin superfamily, nodulin 26 possesses the conserved topology and "hourglass" fold characteristic of this protein family (Walz et al., 2009). This conserved topology consists of six transmembrane domains, five interhelical loops, and hydrophilic N-terminal and C-terminal exposed to the cytosolic side of the membrane (Fig. 69.2). Aquaporin proteins also possess two highly conserved "asparagine–proline–alanine (NPA)" motifs in loops B and E, which form small α-helices that fold back and pack with the six transmembrane α-helices to form the channel pore for substrate transport.

Unlike animal and microbial aquaporins, the evolution of higher plants was accompanied by a diversification of the number and complexity of genes encoding aquaporin proteins (Gupta and Sankararamakrishnan, 2009; Chaumont et al., 2001; Johanson et al., 2001; Sakurai et al., 2005). Based on computational modeling and structural analysis, the hourglass fold is conserved in plant MIPs, but the pore regions show unprecedented structural diversity (Wallace and Roberts, 2004; Bansal and Sankararamakrishnan, 2007; Gupta and Sankararamakrishnan, 2009) that has resulted in

the acquisition of multiple transport activities that transcend the traditional "aquaporin" paradigm (Maurel et al., 2008; Ludewig and Dynowski, 2009; Hachez and Chaumont, 2010).

NIPs are a subfamily of structurally related plant-specific channels of the aquaporin superfamily that have the conserved hourglass template, but which have three distinct "pore subgroups" (Rouge and Barre, 2008). NIPs within each pore subgroup exhibit distinct transport selectivity for various transport substrates including water, ammonia, polyols, organic acids, and metalloid compounds (e.g., boric acid and silicic acid) (Rivers et al., 1997; Dean et al., 1999; Choi and Roberts, 2007; Takano et al., 2006; Li et al., 2011; Ma et al., 2006; Niemietz and Tyerman, 2000; Hwang et al., 2010).

69.3 SYMBIOSOME MEMBRANE NODULIN 26: TRANSPORT PROPERTIES

Ever since its discovery as an aquaporin-like protein, the physiological role of nodulin 26 has been subject to debate. Functional analyses in *Xenopus* oocytes and in proteoliposomes and SM vesicles show that nodulin 26 possesses aquaporin activity (Rivers et al., 1997; Dean et al., 1999). However, these analyses show that nodulin 26 is a relatively poor water channel, with a single-channel conductance for water (P_f) that is 30-fold lower than robust water-selective aquaporins such as mammalian AQP1. However, because of its high SM concentration, nodulin 26 is responsible for the unusually high water permeability of the SM. The SM osmotic water permeability (P_f = 0.05 cm/s), is 50-fold higher than normal membrane bilayer diffusion rates (Rivers et al., 1997). Considering the fact that the symbiosome is the major organelle in the nonvacuolated infected cell, this high water permeability could serve as an osmoregulatory role in cell cytosolic volume homeostasis and osmotic adjustment, similar to the role of the central vacuole of other plant cells.

The water permeability of nodulin 26 is enhanced by phosphorylation of Ser 262 within the cytosolic C-terminal domain of the protein (Guenther et al., 2003). Phosphorylation of nodulin 26 is catalyzed by a Ca^{2+}-dependent protein kinase (CDPK) that is colocalized to the SM (Weaver et al., 1991) and is triggered by water-deficit stress. Given the sensitivity of the nodule and nitrogen fixation to osmotic stress (Del Castillo and Layzell, 1995; Marino et al., 2007; Gil-Quintana et al., 2013), another potential function could be the adaptation of infected cells and endosymbionts to osmotic challenge.

Although nodulin 26 possesses a low intrinsic aquaporin activity, it is also permeable to a number of additional uncharged solutes of potential importance to the symbiosis. For example, similar to mammalian aquaglyceroporins, nodulin 26 shows permeability to small uncharged polyols such as glycerol (Rivers et al., 1997; Dean et al., 1999). While the symbiotic significance of this activity is not clear, rhizobia can utilize glycerol as a carbon source and contain glycerol-inducible ATP-glycerokinase and glycerophosphate (Arias and Martinez-Drets, 1976).

More recently, it has also been demonstrated that nodulin 26 facilitates the transport of NH_3 (Hwang et al., 2010). Measurements of single-channel permeabilities of nodulin 26 reconstituted into proteoliposomes by using stopped-flow fluorometric techniques showed that nodulin 26 possesses a fivefold higher preference for NH_3 as a substrate compared to water (Hwang et al., 2010).

69.4 EFFLUX OF FIXED NITROGEN FROM THE SYMBIOSOME

The ammoniaporin activity of nodulin 26 is of particular interest, given the nature of the SM as a barrier for the efflux of fixed nitrogen. The process of fixed NH_3 and NH_4^+ release from the symbiosome to the plant cytosol for assimilation is complex and could occur by two distinct pathways (Fig. 69.3).

69.4.1 Pathway 1: Directional NH_4^+ Release via a Nonselective Cation Channel

Patch clamp analysis of soybean (Tyerman et al., 1995) and *Lotus japonicus* (Roberts and Tyerman, 2002) symbiosomes has revealed the presence of a voltage-activated nonselective cation channel (NSCC), which is permeated by ammonium, sodium, and potassium ions (Tyerman et al., 1995).

Although the molecular identity of this channel is unknown, it possesses the properties of inwardly rectified K_{ir} channels of mammalian systems (Hibino et al., 2010) and shows unidirectional (i.e., rectified) transport toward the cytosol due to gating by divalent cations or polyamines on the cytosolic side of the membrane (Whitehead et al., 1998, 2001; Roberts and Tyerman, 2002; Obermeyer and Tyerman, 2005). Thus, NH_4^+ in the symbiosome would be transported in a unidirectional manner toward the cytosolic compartment by the NSCC without significant backward flux.

Regulation of gating is mediated by the transmembrane voltage potential ($V_{0.5}$ = −60 to −80 mV), as well as by the symbiosome concentration of NH_4^+ ions (Whitehead et al., 1998; Roberts and Tyerman, 2002; Obermeyer and Tyerman, 2005). This channel is tightly regulated and opens only in response to a voltage gradient ($\Delta\psi$) across the membrane. The $\Delta\psi$ is established by a proton pumping SM-ATPase (Udvardi and Day, 1989), which hydrolyzes ATP and simultaneously transports a proton from the plant cytosol into the symbiosome. This establishes a "proton motive force,"

which consists of two components: an electrical component ($\Delta\psi$) and a chemical component, which is the pH gradient (ΔpH) caused by a higher H^+ inside the symbiosome.

69.4.2 Pathway 2: Facilitated Transport of NH_3 by Nodulin 26

A protein-based facilitated pathway for flux of uncharged NH_3 across the SM was originally described by Niemietz and Tyerman (2000). Based on the properties of nodulin 26 as an ammoniaporin (Hwang et al., 2010) and its high concentration on the SM, it is likely to account for this facilitated pathway observed in purified SM vesicles. This pathway shows no dependence on $\Delta\psi$ and would transport NH_3 in a bidirectional manner. Therefore, the direction of transport would depend on the concentration gradient of NH_3 across the SM.

The relative contribution of the NSCC and nodulin 26 pathways to the overall process of fixed nitrogen efflux depends on several factors: (i) the concentration gradient of NH_4^+ and NH_3 between the symbiosome space and the cytosol; (ii) The ΔpH between the symbiosome space and cytosolic compartments; and (iii) The resting voltage potential ($\Delta\psi$) of the SM. These parameters are controlled by the activity of the SM H^+-pumping ATPase. For example, highly active H^+-ATPase hyperpolarizes the SM (high $\Delta\psi$), resulting in the opening of the NSCC and acidification of the symbiosome space (elevated ΔpH). Based on mathematical modeling (unpublished), these conditions would favor efflux of fixed nitrogen through the open NSCC over the nodulin 26 pathway (Fig. 69.3a). In contrast, low ATPase activity would result in reduced SM potential (low $\Delta\psi$) and a decreased ΔpH, which would lead to the closure of the NSCC that would favor NH_3 efflux via nodulin 26 (Fig. 69.3b).

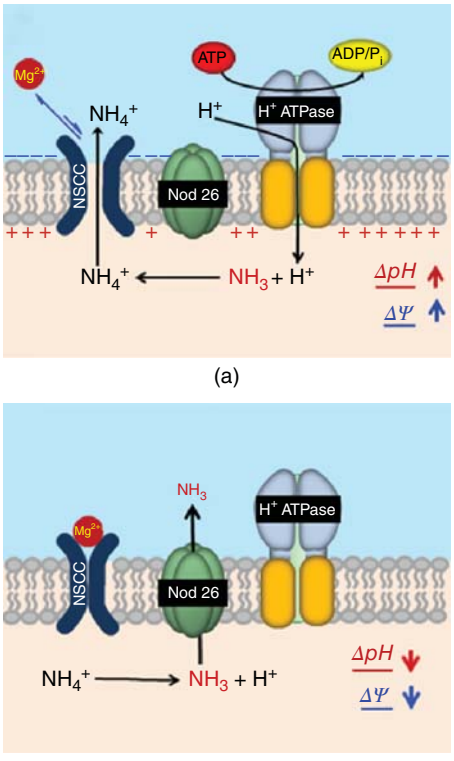


Figure 69.3 Two pathways for fixed nitrogen efflux. (a) Voltage-dependent efflux of ammonium ion through a nonselective cation channel (NSCC). This pathway is favored under high H^+-ATPase activity that leads to a proton motive force across the SM. At negative potentials, the gating particle (Mg^{2+} or polyamines) dissociates from the NSCC resulting in channel opening. The acidification of the symbiosome space also increases the $[NH_4^+]/[NH_3]$ ratio, which would also favor the NSCC pathway. (b) Facilitated diffusion and uncharged ammonia through the nodulin 26 aquaporin-like channel. This pathway would be favored under conditions of low H^+-ATPase activity, or when the SM is depolarized and the ΔpH is low. Under these conditions, the NSCC is closed and uncharged ammonia movement through nodulin 26 would be the pathway for fixed nitrogen movement.

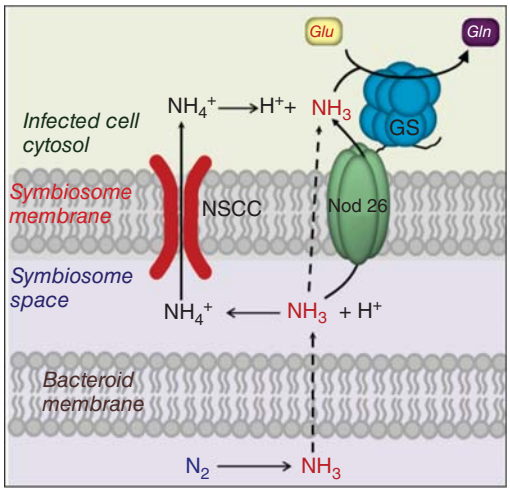
69.5 NODULIN 26, GLUTAMINE SYNTHETASES, AND AMMONIA ASSIMILATION

Upon transport to the cytosol, ammonium ion is assimilated into an organic form, principally via the action of ATP-dependent glutamine synthetase (GS) in the infected cell cytosol (Miflin and Habash, 2002). In soybean, three isoform families of cytosolic GS (designated α, β, and γ) are expressed, with the β and γ isoforms found in nitrogen-fixing nodules (Morey et al., 2002).

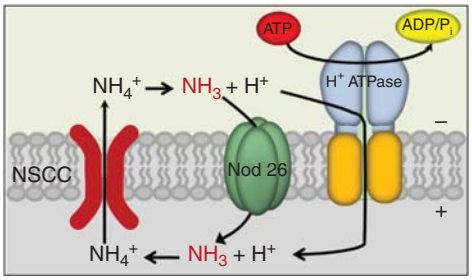
A connection between the transport activity of soybean nodulin 26 and nitrogen assimilation came from the demonstration of an interaction between the β and γ GS isoforms with nodulin 26 via its cytosolic carboxyl terminal domain (Masalkar et al., 2010). This observation is consistent with previous observations with animal aquaporins that show that the carboxyl terminal region is a common site for regulation and interaction with various cytosolic proteins (Yu et al., 2005; Moeller et al., 2010).

The potential significance of nodulin 26/GS association is summarized in Figure 69.4. Nodulin 26 interaction with GS would localize this critical assimilatory enzyme to the symbiosome surface, the site of fixed NH_3/NH_4^+ release into the infected cell cytosol. Direct interaction of GS with nodulin 26 could facilitate rapid assimilation of reduced nitrogen in the form of unprotonated NH_3 transported through the nodulin 26 channel, potentially by substrate channeling (Fig. 69.4).

Additionally, since nodulin 26 is the most abundant SM protein, interaction with GS would increase the local concentration of the enzyme at the symbiosome surface, which



(a)



(b)

Figure 69.4 Model for the nodulin 26/GS interaction and effects on nitrogen efflux and assimilation. (a) Fixed nitrogen within the symbiosome space can be transported as NH_4^+ or NH_3 by the NSCC or nodulin 26 pathways, as discussed in the text. Binding of GS to the C-terminal domain of nodulin 26 serves as a site for rapid assimilation of ammonia traversing nodulin 26. Additionally, binding of GS to the symbiosome increases its local concentration, further facilitating the assimilation of fixed nitrogen released by other pathways as well. (b) Ammonia futile cycling through the SM. H^+-ATPase mediates the movement of protons from the cytosol to the symbiosome space generating a proton gradient leading to polarization of SM and activation of the NSCC. This channel directionally transports NH_4^+ into the cytosolic compartment where alkaline conditions lead to the release of H^+, with NH_3 potentially re-entering the symbiosome space through nodulin 26. Rapid assimilation of NH_4^+ via GS would maintain cytosolic NH_4^+ levels at low concentrations, thereby preventing this potential metabolite cycling. (Adapted from Masalkar et al. (2010) with permission.)

would enhance the rate of fixed nitrogen assimilation transported by other efflux pathways such as the NSCC.

Another potentially important outcome of this interaction is that it may aid in preventing the toxic effects of cytosolic accumulation of ammonia (Fig. 69.4b). The toxicity of cytosolic ammonia in higher plant systems is potentially the result of "futile cycling" (Britto et al., 2001). For example, in the symbiosome, NSCC-mediated efflux of fixed NH_4^+ into the alkaline plant cytosol would result in deprotonation, generating NH_3 that could re-enter the acidic symbiosome space through nodulin 26. The result would be a net transport of a proton from the symbiosome to the cytosol, which would collapse the proton motive force generated by the H^+-ATPase, leading to hydrolysis of ATP and futile cycling. The maintenance of low cytosolic ammonia concentrations, which are estimated to be 50-fold lower than concentrations in N_2-fixing symbiosomes (Streeter, 1989), is critical to prevent futile cycling. The association of GS with nodulin 26 could facilitate rapid nitrogen assimilation, preventing the accumulation of free ammonia in the cytosol.

69.6 SUMMARY

The NIP channels represent a plant-specific channel family with an overall structural conservation of the aquaporin hourglass scaffold, but with pore regions that have diverged so that they have a reduced ability to transport water while acquiring the ability to transport a number of additional metabolic substrates. Nodulin 26, the archetype of the NIP family, which represents one pore subclass (NIP I subclass) (Wallace et al., 2006), is a major component of rhizobia-containing symbiosomes. The protein shows permeability for both water (aquaporin) and NH_3 (ammoniaporin), with the latter being the preferred substrate. A potential role in ammonia efflux is supported by the interaction of the nitrogen assimilatory enzyme GS with nodulin 26. While the biochemical and biophysical properties of nodulin 26 are well established, several issues regarding the biological role of this symbiosis-specific channel remain unresolved.

1. How does the interplay between the proton pump, the NSCC, and nodulin 26 mediate nitrogen efflux and assimilation. In this regard, the molecular identities of the NSCC and the H^+-ATPase, and how the later is regulated remain to be elucidated.
2. Preliminary data suggest that water and ammonia permeabilities of the SM are conversely regulated by phosphorylation (Niemietz and Tyerman, 2000). Is CDPK-catalyzed phosphorylation a mechanism to regulate osmotic versus metabolic functions of nodulin 26 in response to environmental or metabolic cues?
3. While potential roles for water and ammonia transport for nodulin 26 are demonstrated, assignments of these functions to the protein are difficult because of the multifunctional activity of NIPs (e.g., glycerol and metalloid transport). Physiological analysis of loss-of-function mutants may present an approach to elucidate which of these multiple transport activities are relevant to the symbiosis.

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Chapter 70

Leghemoglobins with Nitrated Hemes in Legume Root Nodules

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70.1 INTRODUCTION

Hemoglobins are widespread in all organisms, where they perform multiple and varied functions (see reviews by Vinogradov et al., 2005; Garrocho-Villegas et al., 2007; Angelo et al., 2008; Hoy and Hargrove, 2008). Flavohemoglobins of bacteria and yeast contain globin and flavin adenine dinucleotide (FAD) reductase domains and are involved in nitric oxide (NO) detoxification and protection against nitrosative stress (Angelo et al., 2008; see Chapter 64). In vertebrates, hemoglobin and myoglobin play key roles in efficient O_2 transport and storage and in NO homeostasis, whereas neuroglobin and cytoglobin might be implicated in O_2 supply and act as O_2-consuming enzymes or as O_2 sensors (Pesce et al., 2002). Plants may contain symbiotic, nonsymbiotic, and truncated hemoglobins (Garrocho-Villegas et al., 2007; Hoy and Hargrove, 2008), and the three types of globins are expressed in legume nodules (Bustos-Sanmamed et al., 2011). Symbiotic hemoglobins include leghemoglobins (Lbs) of legumes and some hemoglobins of actinorhizal plants, and their major function is to facilitate O_2 diffusion within the nodules (Wittenberg et al., 1974). Lbs are present in the cytosol of host cells at concentrations of 2–3 mM and maintain a free O_2 concentration of 20–40 nM (Becana and Klucas, 1992). This range of O_2 concentration permits an adequate supply of adenosine triphosphate (ATP) for N_2 fixation and avoids nitrogenase inactivation (Wittenberg et al., 1974). Nonsymbiotic hemoglobins are expressed at nanomolar to micromolar concentrations in most plant tissues, and are classified into two groups based on amino acid sequences, phylogeny, and O_2-binding properties. Class 1 display extremely high affinity for O_2 and participate in NO metabolism and in the maintenance of ATP production under hypoxia (Hebelstrup et al., 2007; see Chapter 64). Class 2 hemoglobins have similar O_2 affinities to Lbs and unknown function (Trevaskis et al., 1997) but, in *Arabidopsis thaliana*, the class 1 and 2 hemoglobins together are required for normal growth (Hebelstrup et al., 2006). Class 3 or truncated hemoglobins have a 2/2 α-helical sandwich secondary structure instead of the canonical 3/3 structure of other hemoglobins, and are present in bacteria, protozoa,

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and plants (Vinogradov et al., 2005). A role for class 3 hemoglobins has not been conclusively demonstrated yet, although it has been suggested that they may be involved in NO detoxification (see Chapter 64) and, in legumes, in the suppression of the defense response during symbiosis (Wittenberg et al., 2002; Hoy and Hargrove, 2008).

In most legume nodules, Lbs exist as multiple components or isoproteins whose relative proportions vary with age and stress conditions (Fuchsman and Appleby, 1979). In soybean nodules, there are four major components (a, c_1, c_2, c_3), encoded by different genes, and four minor components (b, d_1, d_2, d_3), originated by posttranslational modification (Fuchsman and Appleby, 1979; Whittaker et al., 1979). The regulatory pathways of Lb biosynthesis have been extensively studied (e.g., O'Brian, 1996), but there is virtually no information on the *in vivo* mechanisms of Lb degradation. In animals and plants, the conversion of heme to biliverdins is catalyzed by heme oxygenase (Brown et al., 1990; Baudouin et al., 2004), but can also be carried out nonenzymatically in the presence of ascorbate and O_2 (Lehtovaara and Perttilä, 1978). In plants, biliverdin-like pigments perform important functions in photosynthesis and photomorphogenesis (Brown et al., 1990) and accumulate in senescent nodules (Virtanen and Laine, 1946; Roponen, 1970). Legume nodule senescence is a highly complex and regulated process with potential agricultural and ecological relevance as it limits the functional lifespan of nodules and N_2 fixation (Puppo et al., 2005; Becana et al., 2010).

The green proteins derived from Lb in nodules had not been characterized when we began our work (Navascués et al., 2012). Early studies by Virtanen and Laine (1946) described the presence in legume nodules of a green pigment that originated from Lb. The pigment contained a broken tetrapyrrole ring that still retained the iron. Very different green proteins were isolated from soybean nodules (Jun et al., 1994a). The modified proteins, termed Lba_m and Lbc_m, derive from Lba and Lbc and have identical apoproteins to the precursor Lbs but unknown alterations of the tetrapyrrole ring (Jun et al., 1994a,1994b). Identification of the heme modifications in Lba_m and Lbc_m is important because their proportions relative to the precursor Lbs increase during nodule senescence and because the green Lbs exhibit aberrant binding to O_2 (Wagner and Sarath, 1987). By using a combination of spectroscopic techniques and reconstitution experiments of Lb with mesoheme (heme in which the vinyl groups have been replaced by ethyl groups), we have shown that soybean Lba_m and Lbc_m have a 4-nitrovinyl in their heme groups (Fig. 70.1), and that these modified heme proteins can be generated *in vitro* by exposing Lba and Lbc to NO_2^- (Navascués et al., 2012). Here, we present a summary of the experiments carried out for the identification of nitrated Lbs and discuss relevant mechanisms for nitration of Lb hemes.

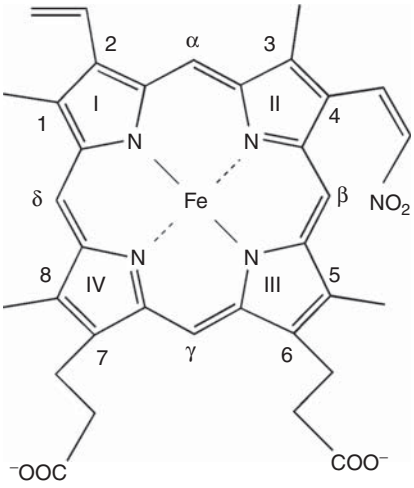

Figure 70.1 Heme structure with the position of the 4-nitrovinyl group. The figure shows an isomer with the NO_2 group in the Cβ atom and in *cis*-configuration. Fisher's numbering of protoporphyrin IX was followed (Source: from (Navascués et al., 2012) with permission).

70.2 METHODS

Soybean plants (*Glycine max* cvs Hobbit or Williams × *Bradyrhizobium japonicum* strains 61A89 or USDA110) were grown under controlled environmental conditions until the late vegetative growth stage. Nodules were harvested in liquid nitrogen and stored at −80 °C.

Soybean Lbs were purified using ammonium sulfate fractionation and chromatography on hydroxyapatite, Sephadex G-75, and DE-52 columns (Jun et al., 1994a). Proteins were subjected to in-gel digestion with trypsin, and identification of the proteins was performed by peptide mass fingerprinting with a matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) instrument (Applied Biosystems; Foster City, USA) as described (Casanovas et al., 2009). The molecular masses of Lbs were also determined by MALDI-TOF mass spectrometry (MS).

Hemes and heme proteins were subjected to structural analyses by MS and by nuclear magnetic resonance (NMR) and resonance Raman (RR) spectroscopies as described in detail elsewhere (Navascués et al., 2012). For MS analysis of hemes, low-energy MS^n ($n = 1$–4) was carried out by nanoelectrospray ionization on an LCQ ion trap mass spectrometer (ThermoFisher, San Jose, USA). For NMR analysis of Lbs, ^1H NMR samples were prepared in D_2O containing 50 mM phosphate buffer and 2 mM cyanide to insure complete formation of the ferric Lb-cyano complexes. The entire protein sample available for each Lb was used (~1 mM of Lba and ~0.2 mM of Lbc_m). NMR spectra were collected at 30 °C with the proton chemical shifts referenced to residual water. 1D and Nuclear Overhauser Enhancement

SpectroscopY (NOESY) spectra were obtained on a Bruker DRX-500 NMR spectrometer operating at 499.38 MHz proton Larmor frequency. RR spectra were recorded using a Jobin-Yvon U1000 spectrometer, equipped with a liquid nitrogen-cooled charge-coupled device (CCD) detector (Spectrum One, Jobin-Yvon, France). Excitation at 413.1 nm (50 mW) was provided by an Innova Kr^+ laser (Coherent, Palo Alto, USA).

The Lb*a* apoprotein was obtained by the acid-butanone method (Ascoli et al., 1981). After neutralization of the aqueous phase with phosphate buffer (pH 7.0), the apoprotein was incubated overnight with a twofold excess of protoheme or mesoheme, dialyzed, and nitrated. For time-course studies of nitration, heme proteins (150–200 μM) were treated with $NaNO_2$ (200 mM) in 50 mM phosphate buffer (pH 5.5 or 7.0) for 2–48 h at room temperature. The mixtures were dialyzed, concentrated, and resuspended in water (isoelectric focusing (IEF) analysis) or in 10 mM NH_4HCO_3 (MS analysis).

70.3 RESULTS

70.3.1 Purification of Lbs and Their Modified Forms

The major Lb components and their green derivatives were purified from soybean nodules by ammonium sulfate fractionation, conventional chromatography, and IEF. All bands containing Lbs were excised from the gels, and the proteins were eluted and analyzed by MALDI-TOF/MS. The molecular masses of the apoproteins of Lb*a*, Lbc_1, Lbc_2, and Lbc_3, as well as those of their respective modified forms, were found to be 15,241, 15,256, 15,393, and 15,451 Da, respectively, which matched ±1 Da those predicted from the amino acid sequences excluding the initial Met. We also purified two fractions containing the Lb*d* and Lbd_m components. The molecular masses of the apoproteins of Lbd_1, Lbd_2, and Lbd_3 were found to be 15,299, 15,436, and 15,492 Da, which exceed by 42 ± 1 Da those of Lbc_1, Lbc_2, and Lbc_3, respectively. This mass difference is consistent with the presence of an N-terminal acetylation. Also, the apoproteins of the Lbd_m derivatives have identical molecular masses to those of the parent proteins. It can be thus concluded that all four minor Lb components of soybean arise from the major components by N-terminal acetylation, and that all the green Lb derivatives are affected in the hemes and not in the globins.

70.3.2 Structural Elucidation of Modified Hemes

Purified Lb*a*, Lb*c*, and Lbc_m from soybean nodules were used for comparative structural analyses of the protoheme and the modified heme by using UV–visible, MS, NMR, and RR spectroscopies. Ferric Lbc_m exhibits a Soret band at 389 nm with a shoulder at 436 nm and a charge-transfer absorption band at 615 nm. The pyridine hemochrome spectrum of Lbc_m was identical to that of Lba_m (Jun et al., 1994a), with prominent absorption bands at 553 nm (α band) and 522 nm (β band) and a new peak at 580 nm. Therefore, the heme of Lbc_m is not broken and retains the capacity for ligand binding, but it is chemically modified on the tetrapyrrole ring.

To identify this modification, hemes were subjected to MS^n fragmentation. Because the isolated modified hemes were relatively unstable, the whole proteins were directly subjected to MS analysis. The hemes of Lb*a*, Lb*c*, and Lb*d* had an *m/z* 616, as expected for protoheme, whereas those from Lba_m, Lbc_m, and Lbd_m had an *m/z* 661. High-resolution MS of these molecular ions proved that the difference of 45 Da was due to the insertion of an NO_2 group. The molecular ions were extensively fragmented (MS^2 to MS^4), and the elemental compositions of the most relevant fragments were elucidated by high-resolution MS. These analyses revealed that one propionic group, at least the α-carbon and carboxyl of the other propionic group, and at least three methyl groups of the tetrapyrrole were intact in the modified hemes. The fragmentation patterns of the Lba_m, Lbc_m, and Lbd_m hemes were identical, thus confirming, together with the Soret–visible spectroscopy data, that all of them contain an NO_2 group.

Further structural information on the modified hemes was obtained by ^1H NMR spectroscopy using the ferric-cyano complexes of Lb*a* (standard for comparison) and Lbc_m. The Lb*a* sample was found to have a 1D ^1H NMR spectrum with identical proton signals to that already published (Trewhella and Wright, 1980). However, the sample of Lbc_m protein was relatively small and composed of a mixture of Lbc_{1m} and Lb$c_{2m} + c_{3m}$, and hence it was not possible to assign as many of the heme resonances of these Lbc_m isoproteins. The chemical shifts of all of the heme methyl groups of the two major species of the Lbc_m sample were changed by the heme modification, in part because of a distortion of the heme seating by 7°. By contrast, no shifts were detected for the 2-vinyl group or for most of the protons of the 6- and 7-propionate groups, and it was concluded that the modification of the heme appears to be at the 4-vinyl substituent (see details in Navascués et al., 2012). However, none of the protons of the 4-vinyl group of Lbc_m could be identified, and thus we had to complement our study with RR spectroscopy and reconstitution experiments.

The RR spectra of ferric Lb*a* and Lbc_m were compared (see details in Navascués et al., 2012). The high-frequency regions of the RR spectra revealed the binding of an NO_2 group to the protoheme of Lbc_m, with a signature at 1320 cm^{-1}, specific of a nitroaromatic group. In the mid-frequency and low-frequency RR spectra, the frequencies of

modes involving the peripheral vinyl and methyl groups are significantly modified, further indicating the presence of an NO_2 group in a vinyl.

70.3.3 Reconstitution of Lbs with Mesoheme and *In Vitro* Nitration

To verify that the nitrated heme originated by a substitution of a proton by NO_2 on a vinyl group, we prepared the apoLbs, reconstituted the holoproteins with protoheme or mesoheme, and nitrated the reconstituted proteins (Fig. 70.2).

Nitration of the apoLb reconstituted with protoheme caused formation of green protein products (Fig. 70.2a) with modified visible spectra (Fig. 70.2b) and with heme groups having an *m/z* 661 (Fig. 70.2c). These Lb products showed identical fragmentation patterns to the hemes of the green Lbs from nodules. By contrast, the apoLb reconstituted with mesoheme remained unaffected after the $NaNO_2$ treatment, based on the Soret and visible spectra (Fig. 70.2b), and RR and MS analyses of the protein. The MS analysis showed a molecular ion of *m/z* 620, characteristic of the iron-mesoporphyrin lacking NO_2 (Fig. 70.2c). Taking these results together with the MS, NMR, and RR data, we conclude that the NO_2 group of the modified Lb hemes is on the 4-vinyl, and that several structural isomers are produced by nitration of the protoheme. To confirm the presence of several isomers of Lb hemes, Lb*a* purified from soybean nodules was nitrated with $NaNO_2$ at pH 7.0 or 5.5 at 25°C , and the resulting proteins were resolved on preparative IEF gels (Fig. 70.3). Nitration was faster at pH 5.5 than at pH 7.0, being completed within ~1 and ~2 days, respectively, when ~200 μM Lb and ~200 mM NO_2^- were employed. At pH 5.5, heme nitration required ~3 days to complete with ~20 mM NO_2^- and was not completed after 5 days with ~2 mM NO_2^-.

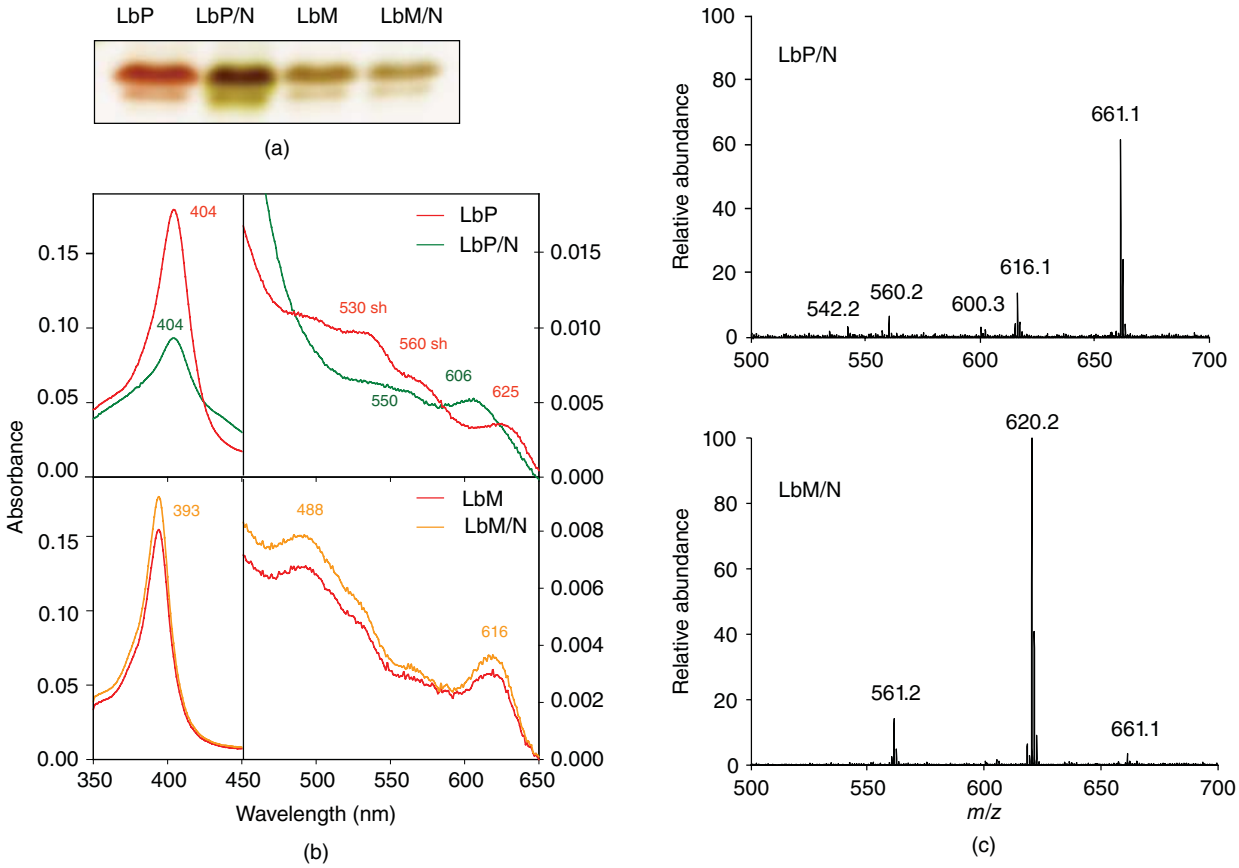

Figure 70.2 *In vitro* reconstitution and nitration of Lb. (a) ApoLb*c* was reconstituted with either protoheme (LbP) or mesoheme (LbM) and treated for 24 h at pH 6.5 with a 1000-fold excess of $NaNO_2$. The products (LbP/N and LbM/N) were loaded on an analytical IEF gel and let to proceed until separation of Lbc_1 (top band) and Lb$c_2 + c_3$ (bottom band). Green nitrated derivatives were formed from the Lb-bearing heme with vinyls (LbP/N) and not from the Lb-bearing heme with ethyl groups (LbM/N). (b) Soret and visible spectra of aliquot samples of the proteins loaded on the gel. Note that LbM and LbM/N have identical spectra, whereas LbP/N is being converted to green derivatives, with a Soret band of lower intensity and a hypsochromic shift of the 625 nm charge-transfer absorption band. (c) Mass spectra of the hemes from Lb*a* reconstituted with protoheme or mesoheme and then nitrated. Note the absence of nitration (*m/z* 620) in the mesoheme (Source: from (Navascués et al., 2012) with permission).

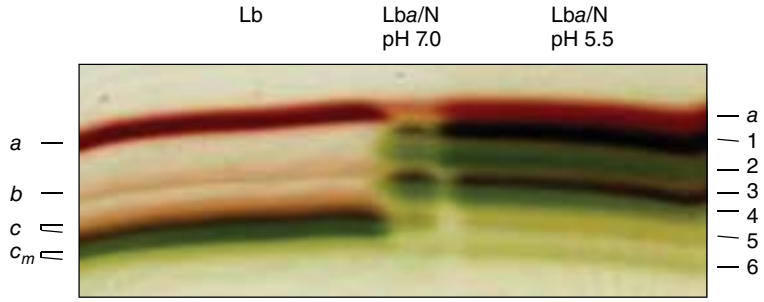

Figure 70.3 Nitration of Lb*a* and separation of the nitrated products on preparative IEF gels. *Left lane*, mixture of Lb*a*, Lb*b*, Lb*c*, and Lbc_m standards. The two Lb*c* protein bands correspond to Lbc_1 and Lb$c_2 + c_3$. *Right lane*, Lb*a* (500 µM) purified from soybean nodules was nitrated with $NaNO_2$ (500 mM) for 48 h in citrate buffer (pH 5.5), yielding six derivatives (Lb*a*N1 to Lb*a*N6). *Center lane*, a similar pattern of Lb*a*N derivatives was obtained when nitration was performed in phosphate buffer (pH 7.0) (Source: from (Navascués et al., 2012) with permission).

Typically, six Lb*a* derivatives were produced (Fig. 70.3). Lb*a*N6 was less abundant and could not be studied further. All other derivatives had pyridine hemochromes with a 580 nm band that is absent in unmodified Lbs. The ferric forms of the Lb*a* derivatives had Soret bands at 391–403 nm with shoulders at 433–436 nm, as well as a charge-transfer band at 615 nm. The Soret and RR spectra of Lb*a*N4 showed the closest match to those of Lba_m or Lbc_m, and we thus conclude that Lb*a*N4 has an identical modified heme to the modified Lbs.

All Lb*a*N derivatives had hemes with *m/z* 661 and identical MS^n fragmentation profiles. Likewise, all the apoLb*a*N derivatives were found to have a molecular mass of 15,240 Da and hence do not bear any modification in their amino acid residues. Consequently, the *in vitro* nitration of Lbs with excess NO_2^- can reproducibly generate the modified Lbs found in nodules, as well as several isomers of nitrated hemes.

70.3.4 Involvement of RNS in Heme Nitration

Both NO_2^- and NO cannot nitrate proteins directly, whereas other reactive nitrogen species (RNS) derived therefrom can do it *in vitro* and *in vivo*. These oxidant and nitrating RNS include peroxynitrite ($ONOO^-$), nitrogen dioxide (NO_2), and nitronium (NO_2^+) salts (Olah et al., 1982; Brennan et al., 2002; Nicolis et al., 2004). To investigate the nature of the RNS involved in heme nitration, *in vitro* experiments were carried out with purified soybean Lb*a*. This task is complicated because $ONOO^-$, when present as peroxynitrous acid (ONOOH), can undergo homolytic cleavage to NO_2 and hydroxyl radical ($^{\bullet}OH$), and because nitrous acid (HNO_2) can give rise to NO_2^+. Addition of 10 mM cyanide completely prevented nitration, indicating that the heme iron is participating in the reaction. To examine whether $ONOO^-$ was the nitrating agent, we used 3-morpholino-sydnonimine (SIN-1). This compound spontaneously decomposes to produce NO and superoxide anion radicals ($O_2^{-}\bullet$), which then react with each other to form $ONOO^-$. Thus, SIN-1 can mimic a slow exposure of the protein to $ONOO^-$. Incubation of Lb*a* with 0.5–1 mM SIN-1 at pH 5.5 or 7.0 for up to 4 h did not nitrate the heme, excluding any contribution of free $ONOO^-$ to nitration. Likewise, an exogenous supply of superoxide dismutase (50–100 µg) or catalase (50–100 µg) did not prevent nitration, and therefore production of $O_2^{-}\bullet$ or H_2O_2 outside the protein is not involved in the reaction. Addition of 30–100 µM H_2O_2 did not promote nitration, confirming that peroxide is apparently not required. By contrast, incubation of Lb*a* with 1 mM desferrioxamine (DFO) for 2–48 h inhibited nitration substantially (Fig. 70.4). DFO is a natural iron chelator commonly used to establish the dependence of biological reactions on free $Fe^{2+/3+}$ ions, but can also intercept free radicals (Bartesaghi et al., 2004). To gain information on the inhibitory effect of DFO and the role of metals on Lba nitration, we used ferrioxamine (FO) (1 mM), prepared by equimolar mixing of DFO and Fe^{3+} ions, and two powerful metal chelators, diethylenetriamine pentaacetic acid (1 mM), and Chelex resin (5 mg). Neither FO (Fig. 70.4) nor the other two compounds had any effect on heme nitration when added to the heme protein prior to NO_2^- and, therefore, free metal ions are involved in the reaction.

70.4 DISCUSSION

Green pigments and nitrated derivatives have been generated *in vitro* from animal and plant heme proteins. In plants, horseradish peroxidase was found to be nitrated on the vinyl groups (Wojciechowski and Ortiz de Montellano, 2007), and a green derivative of Lb was produced by the oxidative attack with H_2O_2 (Moreau et al., 1995). The latter authors proposed that the green Lb species was formed, at least in part, by heme–globin cross-linking. We failed to detect such compounds *in vivo* but instead found that the green Lbs of soybean originated by heme nitration. Spectroscopic and reconstitution analyses of the heme protein revealed that the NO_2 group is on the 4-vinyl (Fig. 70.1). The modified

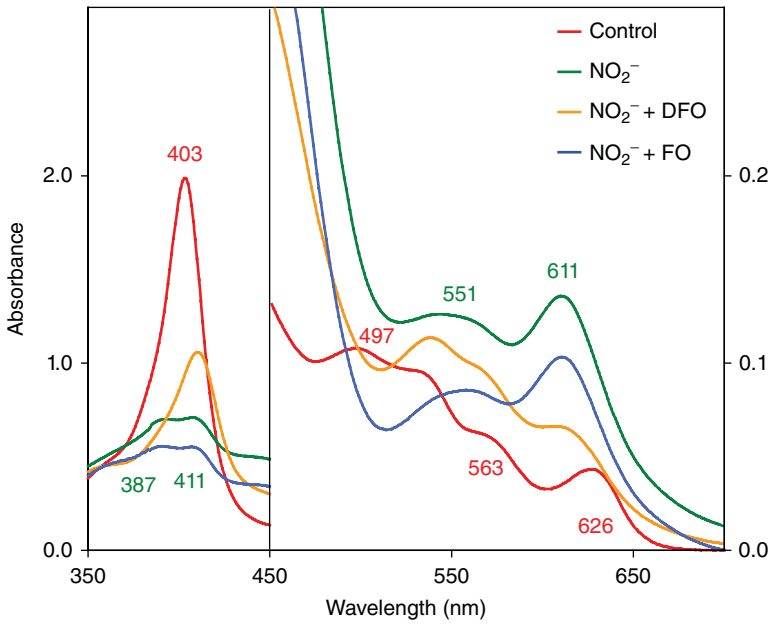

Figure 70.4 Inhibitory effect of DFO on nitration of Lb*a* after 48 h. Ferrioxamine (FO) had only a slight effect. The reaction mixtures contained 50 mM Na-phosphate buffer (pH 7.0), 150 µM Lb*a* and, where indicated, 200 mM $NaNO_2$, 1 mM DFO, or 1 mM FO. Cyanide (10 mM) formed ferric cyanocomplexes that completely suppressed nitration. By contrast, this reaction was not inhibited by addition of superoxide dismutase, catalase, diethylenetriamine pentaacetic acid, or Chelex resin. The reaction was not inhibited with 1–5 mM *p*-hydroxyphenylacetic acid, a more hydrophilic analog of Tyr, which is a good scavenger of the NO_2 radical.

Lbs were synthesized *in vitro* by exposing the proteins to excess NO_2^-. These findings are fully consistent with a study showing that nitration of the horseradish peroxidase heme occurs preferentially on the 4-vinyl rather than on the 2-vinyl (Wojciechowski and Ortiz de Montellano, 2007). Early studies had shown the presence in soybean nodules of three Lba_m derivatives with virtually identical Soret–visible spectra (Jun et al., 1994b). Here, we also found different Lba_m products from *in vitro* nitration of Lb*a* and propose that they are isomers differing in the position of the NO_2 group on the 4-vinyl, such as the α- or β-carbons and/or *cis*- or *trans*-configuration (Fig. 70.1).

To gain insights into the nature of the nitrating molecules and of the nitration mechanisms involved in the production of green Lbs, we used RNS scavengers and releasing compounds, antioxidants, and metal chelators (Figs. 70.4 and 70.5). Nitration of Lb requires binding of NO_2^- to the heme because it was inhibited by cyanide. The reaction is strongly pH dependent, which points out the implication of a nitrating agent derived from HNO_2 rather than from

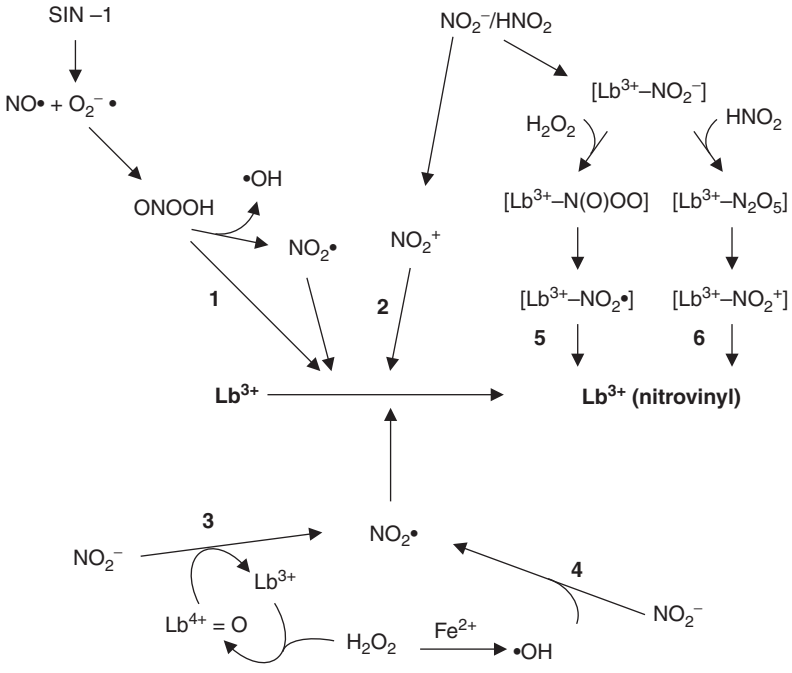

Figure 70.5 Mechanisms that may be operative in the nitration of Tyr residues and/or heme groups of Lb and other heme proteins. Experiments designed to test these pathways are described in the text. *Additional abbreviations:* Lb^{3+}, ferric Lb; Lb^{4+}=O, ferryl Lb; Lb^{3+} (nitrovinyl), ferric Lb bearing a vinyl-bound NO_2 group in the heme (Source: from (Navascués et al., 2012) with permission).

NO_2^- itself. The various mechanisms that can be potentially operative for heme or Tyr nitration in Lbs are depicted in Figure 70.5 and numbered from 1 to 6. Briefly, ***pathway 1*** requires formation of ONOOH outside the protein, but this mechanism can be excluded because SIN-1 did not nitrate the Lb heme and superoxide dismutase and catalase did not prevent nitration. ***Pathway 2*** entails an oxidative attack of Lb by NO_2^+ generated by decomposition of HNO_2 outside the protein, but this mechanism can also be excluded because addition of 1–10 mM nitronium tetrafluoroborate (NO_2BF_4) did not elicit heme nitration. ***Pathways 3*** and ***4*** involve oxidation of NO_2^- to NO_2 by ferryl Lb (formed by reaction of Lb with H_2O_2) or by $^\bullet OH$ generated *via* Fenton reactions, respectively. These mechanisms can be discarded because nitration did not need H_2O_2 and was not dependent on free metal ions. ***Pathway 4***, which is based on Fenton reactions that require free metals or free hemin, was initially proposed as an alternative to the $ONOO^-$ pathway (Thomas et al., 2002). Exogenous H_2O_2 was not required either for the nitration of horseradish peroxidase heme (Wojciechowski and Ortiz de Montellano, 2007) or for the production of NO_2-Tyr on a plant hemoglobin (Sakamoto et al., 2004). In these two cases, the nitrating agent was proposed to be NO_2 based on the peroxidase activity of the heme proteins. ***Pathways 5*** and ***6*** were recently proposed for the nitration of myoglobin (Nicolis et al., 2004; 2006) and hemoglobin (Otsuka et al., 2010) with a large excess of NO_2^-. As in our case, these two pathways require binding of NO_2^- to the heme. ***Pathway 5*** entails a subsequent reaction of the [heme–NO_2^-] complex with H_2O_2 to form a heme-bound peroxynitrite (heme–N(O)OO] species (Nicolis et al., 2004; 2006). However, ferric Lb very rapidly isomerizes $ONOO^-$ to NO_3^-, and hence ***pathway 5*** is unlikely to play a major role in Lb heme nitration (Herold and Puppo, 2005). This pathway would require formation of H_2O_2 inside the heme crevice and probably decomposition of the protonated species [heme–N(O)OOH] to NO_2. ***Pathway 6*** proposes that N_2O_5 is an intermediate (Otsuka et al., 2010). In this case, the [heme–NO_2^-] complex would react with another molecule of HNO_2 giving rise to N_2O_5, which in turn would decompose to NO_2^+ and NO_3^-. Our findings that HNO_2 is the precursor of the nitrating agent and that a [heme–NO_2^-] complex is a prerequisite for nitration are fully consistent with this hypothesis. We propose that nitration is mainly a result of an electrophilic attack on the vinyl by the NO_2^+ generated from HNO_2 inside the heme pocket according to ***pathway 6***, although we cannot discard the simultaneous formation of NO_2 by ***pathway 5*** as mentioned earlier.

The deoxyferrous and oxyferrous forms of Lb are predominant in nodules, but ferric Lb (Lee et al., 1995) and ferrous nitrosyl Lb (Lb-NO) (Mathieu et al., 1998) have been detected also in intact nodules. Ferric Lb can arise from autoxidation of oxyferrous Lb or from the reaction between NO and oxyferrous Lb, in which the heme protein would act as an NO dioxygenase producing NO_3^- (Herold and Puppo, 2005). In nodules, NO_2^- and NO are produced as a result of the nitrate reductase activities in the cytosol and bacteroids (Becana et al., 1989; Meakin et al., 2007; Horchani et al., 2011; see also Chapter 64). An NO synthase-like activity, initially reported in lupin nodules, could also be a contributing source of both RNS (Cueto et al., 1996). In soil-grown legumes, nitration reactions are likely to occur because Lb may be exposed to NO_2^- over weeks or months and because the pH decreases to 5.5 during nodule senescence (Pladys et al., 1988). The presence of a nitrovinyl in the hemes of green Lbs clearly indicates that nitrating and oxidizing RNS are produced in nodules. These reactive molecules are increasingly produced during aging or under stressful conditions. This is in agreement with the higher ratios of Lba_m to Lb*a* and of Lbc_m to Lb*c*, which were observed with advancing senescence (Wagner and Sarath, 1987). Because the green Lbs may be impaired in O_2 transport (Wagner and Sarath, 1987; Jun et al., 1994b), it will be of interest to determine whether they are generated as unavoidable by-products of Lb-mediated RNS detoxification or play physiological roles still to be discovered in legume nodules.

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Chapter 71

The Role of 1-Aminocyclopropane-1-Carboxylate (ACC) Deaminase Enzyme in Leguminous Nodule Senescence

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71.1 INTRODUCTION

Leguminous plants have the ability to establish a symbiosis with a specific group of bacteria, generally called rhizobia. This interaction requires specific perception of signals between legume and *Rhizobium* to form functional organs or nodules on the host plant, in which the bacteroids fix atmospheric nitrogen and convert it to ammonium as biofertilizer for the legume plant. Thus, rhizobial inoculants can be used instead of chemical nitrogen fertilizer to reduce the cost of legume production, as well as for environment-friendly practices. The nodulation process of the legume–*Rhizobium* interaction has been fairly well elucidated. The legume–*Rhizobium* symbiosis is initiated by the release of plant flavonoids, which are a plant signal that can induce the production of Nod factor (lipochito-oligosaccharide) in specific rhizobia (Stougaard, 2000; see Chapters 50, 51). Subsequently, rhizobia enter the root hairs and penetrate the plant cortex through infection threads, while cortical plant cells are stimulated to divide and allow infection by rhizobia that develop into bacteroids surrounded by a plant-derived symbiosome membrane (Ferguson and Mathesius, 2003; see Chapter 68). To accomplish this beneficial interaction, legume plants must lower the level of plant defense mechanisms that prevent the invasion by bacteria (Colebatch et al., 2004; Shaharoona et al., 2011).

Although the overall infection process and nodule development have been studied for decades, there is little knowledge about legume nodule senescence. The senescence of nodule can be induced by developmental aging of nodules or environmental stress-induced nodule senescence. In response to nodule senescence, nitrogen fixation tends to diminish, in parallel with flowering and pod-filling stages of a plant (Bethlenfalvay and Phillips, 1977; Lahiri et al., 1993). Thus, the plant reduces the high-energy consumption by nitrogenase to provide energy for its reproductive stage. However, studies of male-sterile soybean plants that produce only 15% of the pods versus fertile controls revealed similar nitrogenase reduction rates during the pod-filling stage (Imsande and Ralston, 1982; Riggle et al., 1982). This could indicate that the supply of energy or carbon source resulting from senescence and reduced nitrogen fixation in the nodule did not contribute to pod filling. On the other hand, exogenous application of nitrogen during pod filling increases both yield and seed protein content of legumes (Merbach and Schilling, 1980). Therefore, it is possible that a delay in legume nodule senescence, extending the time of nitrogen supply to plant by nitrogen fixation, would increase the yield of legume production (Puppo et al., 2005; see Chapter 64). Thus, the process of delaying nodule senescence, caused by natural aging or by environmental stress induction, should be investigated to prolong nitrogen fixation and

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biofertilizer production for legumes. In this review, the morphology, physiology, and signaling of nodule senescence, as well as the role of 1-aminocyclopropane-1-carboxylate (ACC) deaminase in the delay of nodule senescence will be summarized.

71.2 NODULE SENESCENCE

71.2.1 Morphology of Senescencent Legume Nodule

Legume nodules can be categorized into two types: determinate and indeterminate. Determinate nodules can form on soybean, mung bean, peanut, and some tropical legumes having round-shaped nodule structures because the meristematic cells, which are located in the outer cortex, are nonpersistent. The process of cell division stops during 1–2 weeks after rhizobial inoculation. Another type is indeterminate nodules, which can form with pea, alfalfa, leucaena, and many other legumes having a cylindrical-shaped nodule owing to the small meristematic cells at the tip of the nodule that are continuously dividing (Van de Velde et al., 2006). The nitrogen-fixation zone of determinate nodule occurs homogeneously in the central portion of the nodule, which is composed of the rhizobial infected cells interspersed with some uninfected cells. However, the nitrogen-fixation zone of indeterminate nodules is not homogeneously spread along the whole nodule, but instead occurs only in zone III, while zone I is the apical meristem that does not contain rhizobia, and zone II is the infection zone. Since the different types of nodules have different forms of organization, the nodule senescence pattern is also different. The senescence of determinate nodules develops radially starting from the central area and slowly spreads from the center to the periphery after a few weeks, while the senescence of indeterminate nodule occurs in zone IV that is located proximal to zone III in the mature nodule. The symbiotic relationship is lost in the senescence zone or zone IV of indeterminate nodules and then zone V develops proximal to the senescence zone. There is no presence of symbiosome features in this zone, while the rhizobia are converted to saprophytic intracellular rhizobia (Timmers et al., 2000; Puppo et al., 2005).

Senescence of legume nodules is visible by a color change in the nitrogen-fixation zone. Since the leghemoglobins decline when the nodule is entering the senescence stage, the pink color of the nitrogen fixation zone that is associated with functional leghemoglobin protein will change to a green color in indeterminate nodule due to the degradation of the heme moiety in leghemoglobin (Roponen, 1970). It is similar to determinate nodules, in which the color of symbiosome changes from a pink color to a gray color as shown in Figure 71.1a. Moreover, changes on the cellular level are also observed. It has been reported that

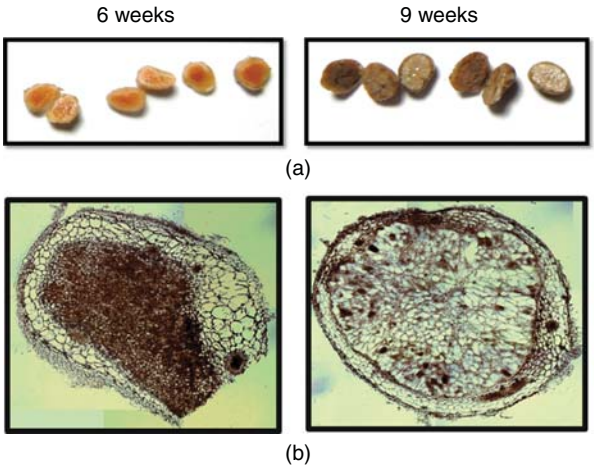

Figure 71.1 Root nodule of mung bean (*Vigna radiata* SUT1) observation at 6 and 9 weeks after inoculation. (a) Observation of symbiosome color change. (b) Observation of micromorphology under light microscope.

in senescent nodules, a wide range of proteolytic enzymes are triggered that cause a variety of nodule protein degradation processes, and the cytoplasm becomes less electron dense in the indeterminate nodule of *Medicago* (Pladys and Vance, 1993; Andreeva et al., 1998; see Fig. 71.1b). Moreover, the appearance of vesicles and some membranes occurs inside the senescence nodule, leads to disintegration of host and symbiosome membrane (Timmers et al., 2000; Van de Velde et al., 2006), and finally causes the death of bacteroid and legume nodule cells. Similar to determinate nodules, the senescence of common bean (*Phaseolus vulgaris* L.) nodules shows the collapse of the infection zone, degradation of membrane and cell walls, changes in the number and distribution of starch granules, appearance of protein granules, and disintegration of the central tissue of the nodule (Fernández-Luqueño et al., 2008). Moreover, the number of peroxisomes increases and mitochondria form elongated structures. The symbiosome membrane appears to be the target for degradation during the early senescence process. However, senescence does not occur homogeneously throughout the nodule in determinate nodules (Puppo et al., 2005). Figure 71.1 shows the determinate-type root nodule of mung bean (*Vigna radiata* SUT1) at 6 and 9 weeks after bradyrhizobial inoculation. The senescence nodule at 9 weeks shows a reduced density of symbiosomes and disintegration of the tissue inside the nodule. These nodule senescence morphologies are accompanied with a decrease in nitrogen fixation. Therefore, it is necessary to understand the signals or factors that trigger or are involved in the initiation of nodule senescence in order to extend the period of active nitrogen fixation by delayed nodule senescence and finally the improvement of yield or nitrogen content in the seed. There are two common processes of nodule

senescence, one is developmental nodule senescence and the other is stress-induced nodule senescence. However, the knowledge of nodule senescence has been mostly elucidated with indeterminate-type nodules, and data are still fragmentary at the subcellular level. In this review, we attempt to integrate these data and propose a model for the nodule senescence process.

71.2.2 Developmental Nodule Senescence

As a result of nodule development, large amounts of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide radicals (O_2^-), are produced and accumulate in the nodule due to the high rate of respiration of bacteroids and mitochondria in indeterminate-type nodule (Groten et al., 2005; see Chapter 29). However, the nodule contains a strong antioxidant defense capacity, as well as a high concentration of ascorbate and reduced glutathione (Becana et al., 2000; Matamoros et al., 2003). The nodule also has high activities of antioxidant enzymes, such as superoxide dismutase, catalase, peroxidase, and enzyme for the ascorbate-glutathione cycle (ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase, and glutathione reductase) (Dalton et al., 1986; Gogorcena et al., 1995; Escuredo et al., 1996). This strong antioxidant system is very important for nodule development in order to scavenge the ROS that could reduce the efficiency of nitrogen fixation by the bacteroids. The role of antioxidants in controlling the redox state of the nodule has also been reported in determinate-type nodule. The activity of enzymes of the ascorbate-glutathione cycle and the level of glutathione are important for nitrogen fixation during soybean nodule development (Evans et al., 1999). The exogenous supply of ascorbate to soybean roots was found to increase nodule number and nitrogenase activity (Bashor and Dalton, 1999). Thus, the antioxidant system is needed to maintain the functional nitrogen-fixating nodule during nodule development.

Once aging of the nodule progresses, the nitrogen-fixation efficiency is decreased in parallel with a decrease in the levels of ascorbate and glutathione in the nodule (Groten et al., 2005). Ascorbate has an important role in the regulation of the plant mitotic cell cycle, which is related to cell growth (Potters et al., 2004). Thus, reduction of the ascorbate content may indicate the decline of nodule functioning during aging. On the other hand, glutathione may influence the redox regulation of transcription factors and the protection of DNA against oxidative damage (Matamoros et al., 2013). Thus, a reduction of ascorbate and glutathione may create oxidative stress, which is frequently associated with plant senescence and leads to cell death. Matamoros et al. (2013) also confirm the importance of ascorbate and glutathione in nodule senescence at the subcellular level. They found that the marked decline in glutathione reduced the capacity to regenerate ascorbate and also upregulated the alternative oxidase and manganese superoxide dismutase in the mitochondria of common bean nodule (*P. vulgaris* L. cv Contender) during early senescence. The early decrease in glutathione in mitochondria in mature nodules may be due to degradation by age (Clemente et al., 2012). Thus, nodule mitochondria are an early target of oxidative modifications, which produce oxidized lipids and proteins, and these molecules may act as signal molecules for the aging process (Matamoros et al., 2013). The occurrence of oxidative stress in aging nodules has been found in soybean (Evans et al., 1999), lupin (Hernandez-Jimenez et al., 2002), and bean (Loscos et al., 2008). However, the accumulation of oxidative stress due to H_2O_2 and ROS is not observed in pea nodules (Groten et al., 2005). Thus, it is possible that there may be some variations in the mechanism of nodule senescence induction in different species of legumes (Puppo et al., 2005). In addition, Matamoros et al. (2013) reported the presence of methionine sulfoxidation (MetSO) as important for regulation of glutamine synthetase (GS) in response to ROS. This posttranslational inactivation of GS may be a direct link between the decline in nitrogen-fixation efficiency and ammonia assimilation in the senescence nodule.

Not only ROS inactivates GS during nodule senescence, but also nitric oxide (NO) may play an important role in the senescence process of determinate nodules, such as soybean (Navascues et al., 2012) and *Medicago truncatula* (Cam et al., 2012; see Chapter 64). It has been reported in *M. truncatula* that NO may be produced by plant nitrate reductase and the mitochondrial electron transport chain (ETC) especially during aging of legume nodules (Horchani et al., 2011). Moreover, denitrification carried out by the bacteria is also a probable source of NO in nodules, but may vary with the bacterial species (Meakin et al., 2007; Sanchez et al., 2010; Horchani et al., 2011). The molecular target of NO in root nodules is GS (Melo et al., 2011). Thus, an increase in NO level could lead to GS inhibition and a subsequent decrease in ammonium assimilation by the plant, leading to nodule senescence. Bacterial proteins may also be targets of NO. Nitrogenase from soybean root nodules has also been shown to be inhibited by NO (Trinchant & Rigaud, 1982; Shimoda et al., 2009; Kato et al., 2010).

In view of the hormonal regulation of nodule senescence, abscisic acid (ABA) has been recognized as an important signal in several types of senescence (Hunter et al., 2004; Buchanan-Wollaston et al., 2005). A lower carbon supply from the plant together with high N-metabolite availability decreases the carbon-to-nitrogen ratio inside nodule. This sugar/N change can be sensed and transduced by an ABA-mediated signaling pathway followed by the activation of the proteolytic activities (Puppo et al., 2005). The increase in nodule ABA activates key proteinase enzymes that are intrinsic to macromolecule degradation during the senescence process. It has been found that the

most abundantly expressed genes during *M. truncatula* nodule senescence are four cysteine (Cys) proteinases (Van de Velde et al., 2006). This family of proteinases is also synthesized in legume and actinorhizal nodules, especially during senescence (Goetting-Minesky and Mullin, 1994; Asp et al., 2004). However, it was found recently that the endoproteinase activity during the senescence of common bean (*P. vulgaris* L. cv Contender) nodules is mainly due to serine proteinases, rather than cysteine proteinases (Matamoros et al., 2013). Another hormone that plays an important role as a part of a signal transduction pathway is ethylene. At early stages of *M. truncatula* nodule senescence, two genes encoding transcriptional factors belonging to the *APETALA/ETHYLENE RESPONSE FACTOR (AP2/ERF)* family were expressed (Van de Velde et al., 2006). These genes are known to be involved in development and disease resistance, as well as in stress responses (Kirch et al., 2003; Gutterson and Reuber, 2004; Yi et al., 2004). Moreover, the upregulation of ERF transcription factors and ethylene biosynthetic genes, such as *S*-adenosyl-Met (SAM) synthetase and 1-aminocyclo-propane-1-carboxylate oxidase, has also been observed in nodule senescence (Van de Velde et al., 2006). The presence of ethylene is usually associated with the induction of ROS signaling to regulate the damaging of cell (Kunkel and Brooks, 2002). There is also cross talk between sugar and N-signaling pathways with ethylene and ABA hormones during the nodule senescence process. It has been found that genes involved in ABA synthesis and signaling and also in ethylene perception are sensitive to sugar and nitrate signaling (Arenas-Huertero et al., 2000; Signora et al., 2001). The lower sugar supply from the plant may activate an increase in ethylene. Therefore, ABA and ethylene may work together in the nodule to activate the senescence process (Puppo et al., 2005). The summary of signaling and the molecules that interact during developmental nodule senescence are presented in Figure 71.2.

71.2.3 Stress-Induced Nodule Senescence

Stress-induced nodule senescence shares several characteristics with aging or developmental nodule senescence, including the decline in N_2 fixation, leghemoglobin, and antioxidant enzymes, as well as the oxidative damage of cell components (Evans et al., 1999; Matamoros et al., 1999; Hernandez-Jimenez et al., 2002; Loscos et al., 2008). However, stress induces nodule senescence development faster than developmental senescence and also presents features of oxidative stress and plant cell death (Puppo et al., 2005). Various types of stress can trigger nodule senescence, such as darkness, water deficiency, salt stress, and cadmium contamination (Gogorcena et al., 1997; Gonzalez et al., 1998; Matamoros et al., 1999; Salah et al., 2013; Balestrasse et al., 2004). As shown in Figure 71.2, stress conditions trigger ethylene production in the plant preceding the senescence in legume nodule. Ethylene is involved in seed germination, root hair development, root nodulation, flower

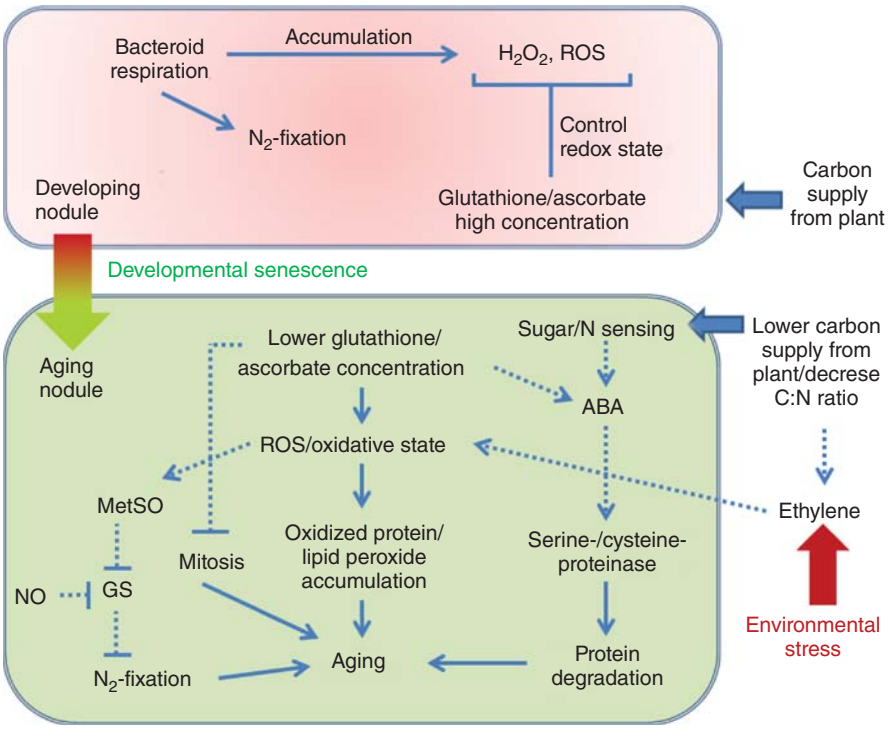

Figure 71.2 A model for developmental and stress-induced nodule senescence process. ABA, abscisic acid; GS, glutamine synthetase; H_2O_2, hydrogen peroxide; MetSO, methionine sulfoxidation; NO, nitric oxide; ROS, reactive oxygen species.

senescence, abscission, and fruit ripening. The production of ethylene is tightly regulated by internal signals during development and in response to environmental stresses. It has been reported that ethylene plays a role in the onset of salt-induced senescence in tomato (Ghanem et al., 2008). Ethylene acts as a signal to activate ROS, which are generated by plant cells in response to abiotic and biotic stresses (Moller et al., 2007). ROS are toxic and able to react with several molecules inside the cell resulting in senescence and cell death (Rivero et al., 2007). The redox state controls nodule metabolism. The decrease in N_2 fixation under abiotic stress is correlated with the decline in antioxidant defense and an increase in ROS levels that lead cells to an oxidative state in senescencing nodule (Gogorcena et al., 1997; Jebara et al., 2005). This correlation is established during both developmental and stress-induced nodule senescence. Another important signaling molecule NO has been observed in the developmental aging of lupin nodules (Evans et al., 1999; Hernandez-Jimenez et al., 2002) and also in stress-induced nodule senescence (Escuredo et al., 1996; Gogorcena et al., 1997; see Chapter 64). An increase in endogenous NO levels leads to a decrease in nitrogen fixation and early nodule senescence. Since NO regulates several physiological processes by affecting gene transcription (Ferrarini et al., 2008; Moreau et al., 2011), it can be hypothesized that NO regulates the expression of genes involved in the senescence program (Cam et al., 2012; see Chapter 64). These signaling molecules, such as ethylene, ROS, and NO, show a relationship between developmental and stress-induced nodule senescence processes (Fig. 71.2). Thus, a lower level of these signaling molecules may delay natural or stress-induced legume nodule senescence, and finally lead to increased nitrogen fixation as well as legume yield. The roles of bacteria-containing ACC deaminase in order to alleviate ethylene production during plant stress and delay nodule senescence are focused on in this review.

71.3 ETHYLENE STRESS AND ACC DEAMINASE ENZYME ACTIVITY IN SYMBIOSIS AND NODULE SENESCENCE

Ethylene is rapidly synthesized in response to external stresses. This kind of ethylene is called stress ethylene, which induces the expression of many stress-associated gene responses (O'Donnell et al., 2001; Saleem et al., 2007). Moreover, it has been reported that ethylene acts as a negative regulator of nodulation in legume plants (Ding and Oldroyd, 2009; Lohar et al., 2009). The exogenous application of ethylene or ethylene-releasing compounds causes abortion of infection threads and suppression of nodulation in legumes (Valverde and Wall, 2005; Tirichine et al., 2006), while application of ethylene inhibitors such as aminoethoxy vinyl glycine (AVG) or silver thiosulfate (STS) increases nodule number (Oldroyd et al., 2001; Tamimi and Timko, 2003), as well as reduces ethylene emission during growth under stress conditions, such as high concentration of nitrate, salt stress, high temperature, and water deficiency (Mann et al., 2002; Tittabutr et al., 2013). However, high concentrations of an ethylene inhibitor cause plant cell damage. Thus, an appropriate concentration of ethylene inhibitor must be used to control the level of ethylene in plant cells, which varies depending on plant species and type of stress condition. Nevertheless, it was found that not all legume species respond to ethylene. Tittabutr et al. (2013) found that mung bean (*V. radiata*) is the most responsive among tested legume species, as well as yard long bean (*V. sinensis*), common bean (*P. vulgaris* L.), and peanut (*Arachis hypogaea* L. Tainan 9). Different varieties of mung bean also respond differently to STS. These observations suggest that the level of ethylene in plant cells is a key factor that influences the nodulation process. Lower level of stress ethylene promotes nodulation as well as plant growth under stress condition.

In the ethylene synthesis pathway, ACC is a precursor. ACC is converted to ethylene by ACC oxidase in higher plant (Yang and Hoffman, 1984; Contesto et al., 2008). Therefore, production of ethylene in plants is directly related to the level of ACC inside the plant tissue. An enzyme that can degrade ACC is ACC deaminase, which is found in bacteria, fungi, and plants (Glick 2005). ACC deaminase catalyzes a cleavage of the cyclopropane ring and leads to the deamination of ACC to α-ketobutyrate and ammonia. At the protein-structure level, ACC deaminase is a multimeric enzyme with a monomeric subunit (molecular mass of approx. 35–42 kDa). It is a sulfhydryl enzyme that utilizes pyridoxal 5-phosphate as a cofactor. Several D-amino acids, such as D-serine and D-cysteine can act as substrates for ACC deaminase, but less efficiently than ACC, while L-serine and L-alanine are effective competitive inhibitors of the enzyme (Glick 2005). ACC deaminase is an intracellular enzyme, which is localized within the cytoplasm (Jacobson et al., 1994). ACC is exuded by plant tissues (Penrose et al., 2001; Penrose and Glick, 2001; Grichko and Glick, 2001) and is taken up by the ACC deaminase-containing microbes (Glick et al., 1998).

ACC deaminase has been detected in several bacteria. However, the activity and regulation of this enzyme are different. The well-known ACC deaminase-producing bacteria *Pseudomonas putida* UW4 (previously *Enterobacter cloacae* UW4) produces ACC deaminase activity equal to 20.48 μmol α-ketobutyrate/mg protein/h (Ma et al., 2003), while other bacteria usually produce low ACC deaminase activity. Some species of rhizobia also have ACC deaminase activity, but the activity level and gene regulation vary significantly in different strains (Duan et al., 2009; Nikolic et al., 2011). Transcriptional regulation of the *acdS* gene

encoding ACC deaminase has been elucidated in *E. cloacae* UW4. The DNA sequence analysis revealed the presence of an *acdR* gene located upstream of the ACC deaminase structural gene (*acdS*) in the opposite direction. The *acdR* encodes an ACC deaminase regulatory protein that has high similarity with a leucine-responsive regulatory protein (LRP). The DNA sequences between *acdS* and *acdR* genes contain several features that are involved in regulation of *acdS* transcription including half of a cAMP receptor protein (CRP)-binding site, an FNR (fumarate-nitrate reduction) regulatory protein-binding site (see Chapter 15), and LRP-binding site (Grichko and Glick, 2000). The expression of *acdR* is under the control of a stronger promoter than the one responsible for transcription of *acdS*. The LRP encoded by *acdR* is recognized as a global regulatory protein, which controls the transcription of genes through the induction of DNA bending, and it is required for *acdS* expression. Moreover, the expression of *acdS* requires ACC to induce transcription. The expression of *acdS* is increased under anaerobic condition, which coincides with the presence of FNR-like regulatory protein box (Li and Glick, 2001). On the other hand, the transcription of *acdS* in *Mesorhizobium loti* MAFF303099 and *B. japonicum* USDA110 is under the control of NifA (Uchiumi et al., 2004; Kaneko et al., 2002). Thus, the pattern of gene organization and transcriptional regulation of the *acdS* gene in different bacteria may result in different levels of ACC deaminase activity.

High ACC deaminase activity has been found to be associated with higher nodulation ability. For example, *R. leguminosarum* containing a higher ACC deaminase activity has been found to be more efficient for pea nodulation (Ma et al., 2003). Similarly, *Sinorhizobium meliloti* containing the ACC deaminase gene (*acdS*) derived from *R. leguminosarum* also showed increased ability to nodulate alfalfa (Ma et al., 2004). Uchiumi and colleagues (2004) showed that inactivation of the *acdS* gene in *M. loti* resulted in a reduced number of nodules on *Lotus japonicus*, compared to the number of nodules formed by the wild-type strain. Tittabutr et al. (2008) evaluated the effect of ACC deaminase on nodulation and growth of *Leucaena leucocephala*. The *acdS* genes were cloned from *Rhizobium* sp. TAL1145 and *Sinorhizobium* sp. BL3 in multicopy plasmids and were transferred to TAL1145. The BL3-*acdS* gene greatly enhanced ACC deaminase activity in TAL1145 compared to the native *acdS* gene. The resulting transconjugants of TAL1145 containing the native and BL3-*acdS* genes formed greater (in number) and bigger nodules and yielded higher root mass on *L. leucocephala* than TAL1145. This result suggests a role for ACC deaminase activity in legume–*Rhizobium* nodulation. Co-inoculation of legumes with rhizobia plant growth-promoting rhizobacteria (PGPR) containing ACC deaminase has also proven to promote nodulation under both normal and environmental stress conditions. Moreover, it was recently found that co-inoculation of mung bean (*V. radiata*) with *Bradyrhizobium* sp. and *Chryseobacterium* sp. ACC3 reduces ethylene production and leads to enhanced nodulation and plant growth under salt stress, water deficiency, and high-temperature stress conditions. Interestingly, the expression of *acdS* of isolate ACC3 was differentially induced when cultured in medium under different stress conditions (Tittabutr et al., 2013). The strong correlation of inducing ACC deaminase activity under stress condition and its activity to reduce the ethylene production in plant suggests that PGPR or rhizobia that produce ACC deaminase should delay senescence of nodules.

Since ethylene is one of important signals that induce both developmental and stress-induced nodule senescence, lower ethylene production in plants after inoculation with rhizobia-containing ACC deaminase may be a way to delay nodule senescence, leading to an increased yield in nodulated legumes. The role of ACC deaminase in nodule senescence has recently been supported by the results of inoculation of mung bean with the mutant of *Sinorhizobium* sp. BL3 defective in ACC deaminase activity (*acdS*$^{-}$), as compared with the transconjugant of BL3 containing *acdS* gene on a high copy number plasmid. The results showed that a defect in ACC deaminase activity affected the nodulation process, plant growth, and nodulation competition negatively, while BL3 with a high copy number of the *acdS* gene delayed nodule senescence when compared with the wild-type strain (unpublished data). As proposed in Tittabutr et al. (2008), increasing ACC deaminase activities in the nodule should decrease ethylene production. It is expected that lowering of ethylene in the root should enhance infection thread elongation and nodule formation in legumes. Enhancing ACC deaminase activities in *Rhizobium* bacteroids inside the nodules reduces ethylene biosynthesis and consequently promotes nodule development (Fig. 71.3). Although it is difficult to assess the effects of increased ACC deaminase activities on nodule senescence and maintenance using indeterminate-type nodules, the effects of increased symbiotic ACC deaminase activities are more visible in mung bean, which produces determinate-type nodules that senescence entirely after a fixed period of nitrogen fixation (unpublished data). Reducing ethylene production may help in nodule maintenance by delaying senescence. These possible benefits associated with enhanced ACC deaminase activities in the nodule should result in an increase in nitrogen fixation and better plant growth promotion.

71.4 CONCLUSION

The senescence of nodules can occur as developmental aging of nodule or environmental stress-induced nodule senescence. In response to nodule senescence, the nitrogen fixation tends to reduce in parallel with damaging of the nodule cell structure through macromolecule degradation. Ethylene plays an important signal for induction of nodule

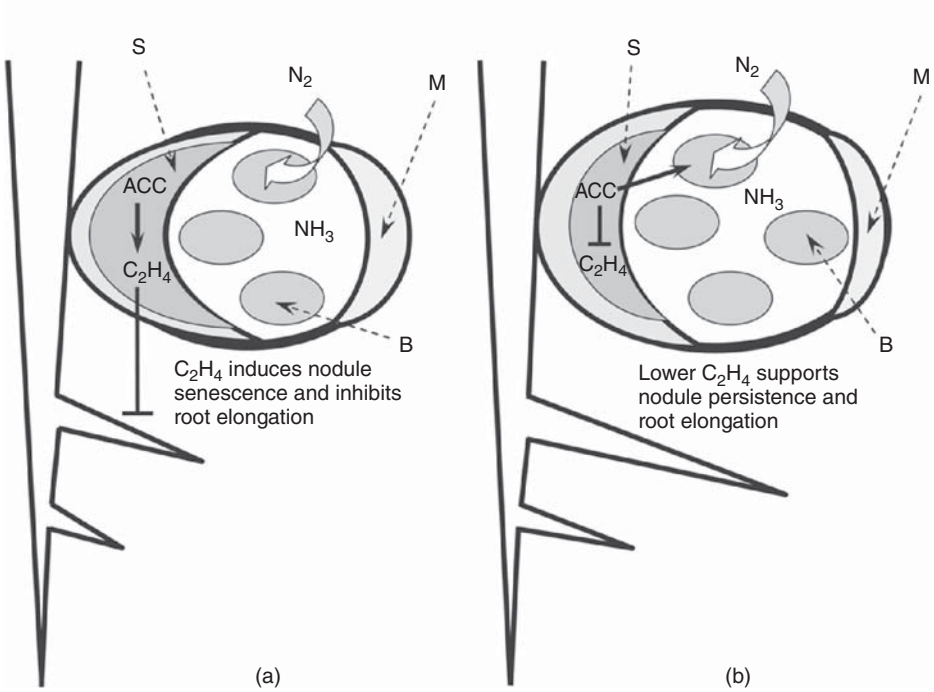

Figure 71.3 Schematic representation of leucaena nodules formed by AcdS$^-$ (a) and AcdS$^+$ (b) rhizobia, highlighting the possible effects of increased ACC deaminase activities on nodule senescence. Ethylene production in nodules formed by AcdS$^+$ rhizobia may be reduced due to ACC deaminase activities. As a result, AcdS$^+$ rhizobia may form bigger nodules with relatively smaller senescence zone and larger nitrogen-fixing bacteroid zone. The increased root size of leucaena seedlings inoculated with AcdS$^+$ rhizobia may be caused by either increased nitrogen fixation by AcdS$^+$ bacteroids or direct sequestering of ACC from the growing roots by AcdS$^+$ rhizobia on the root surface. B, bacteroid; M, meristematic zone; S, senescence zone (Tittabutr et al., 2008).

senescence in both developmental and stress-induced nodule senescence processes by triggering ROS formation and creating an oxidative state inside the cell, leading to accumulation of oxidized biomolecules that activate the aging process. Thus, it is possible to achieve delay of legume nodule senescence by using the strategy of inoculation ACC deaminase-containing rhizobia to reduce the ethylene production during plant growth under both normal and environmental stress conditions. Delayed nodule senescence should extend the time of nitrogen supply to plant by nitrogen fixation and finally increase the yield of legume production.

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Section 13

Microbial “Omics”

Chapter 72

Pool-Seq Analysis of Microsymbiont Selection by the Legume Plant Host

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72.1 INTRODUCTION

The rhizobia are soil organisms that establish a highly successful diazotrophic symbiosis with legumes (see Chapter 4). This symbiosis is critical for agriculture, for the global *N* cycle in general and for modern agriculture in particular (see Chapter 5). One of the key aspects of this symbiosis is its specificity: specific rhizobia nodulate and fix nitrogen in specific legume hosts. The molecular bases for this specificity have been unraveled throughout the last 30 years (see Sections 9 and 10). However, this specificity is not absolute. Some tropical legumes are quite broad in their specificity requirements and are promiscuously nodulated by a large number of different rhizobial species and genera (e.g., *Phaseolus*, siratro, cowpea, or *Leucaena* (Bromfield and Barran, 1990; Perret et al., 2000; Martinez-Romero, 2003; Lima et al., 2009; Cardoso et al., 2012)). Conversely, some rhizobia are able to establish symbioses with very different plants. This is the case of *Sinorhizobium* sp. NGR234 (Stanley and Cervantes, 1991; Pueppke and Broughton, 1999; Perret et al., 2000; see Chapter 32), which uses multiple sets of nodulation and symbiotic genes (Freiberg et al., 1997). Other rhizobia, such as *Sinorhizobium meliloti* (Andronov et al., 1999; Ballard et al., 2005) or *Bradyrhizobium japonicum* (Oh et al., 2000; Koch et al., 2010; Lindemann et al., 2010) can nodulate different hosts depending on a specific genetic complement, often uncovered after mutant screening or isolation of specific strains that are symbiotically active with just some of the hosts.

More subtle mechanisms of adaptation to a given host may be at work in those cases when all (or most) of the isolates of a given rhizobial species can effectively establish a diazotrophic symbiosis with plants of several different genera, often from different habitats and with different lifestyles. This may be the case for *Rhizobium leguminosarum* bv. viciae. Isolates belonging to this biovar establish effective symbioses with legumes belonging to four genera: *Pisum*, *Lens*, *Lathyrus*, and *Vicia*. The last genus, in particular, includes species as diverse as vetch (*V. sativa*) and broad bean (*V. faba*). One set of nodulation and nitrogen-fixation genes, harbored on a symbiotic plasmid, allows successful establishment and development of symbiosis with the different hosts (Surin and Downie, 1989) and, in cross-inoculation experiments, when challenged with any one of the above legume hosts, any *R. leguminosarum* bv. viciae strain is able to establish an efficient symbiosis. However, it has long been hypothesized that different

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rhizobial strains may be more adapted to a specific plant host than others, which may result in selection and enrichment of a specific strain or set of strains by the legume host from those present in a particular soil (see Chapter 19). Evidence for plant-mediated selection of specific rhizobial genotypes from soil populations has been obtained by the Laguerre (Louvrier et al., 1996; Laguerre et al., 2003; Depret et al., 2004) and Young (Palmer and Young, 2000; Mutch and Young, 2004) groups. By using molecular markers and specific polymerase chain reaction (PCR) amplification, these researchers have provided evidence that different plant hosts enrich specific genotypic marker variants of *R. leguminosarum* bv. viciae from those available in the soil.

These studies, however, were necessarily limited by the number and nature of the markers selected, and did not shed any light on the selective forces that might determine the enrichment of a specific genotype. The quick development and widespread availability of cost-effective, next-generation sequencing technologies prompted us to reappraise this problem using genomic and metagenomic approaches. In doing so, we were able to establish a set of methodologies for the genomic study of natural rhizobial populations that may find wider applicability in other systems and problems. In this chapter, we describe these methodologies using the specific problem of *R. leguminosarum* bv. viciae genotype selection by the legume host as an example.

72.2 GENOMICS OF RHIZOBIA

After the ground-breaking reports on genome sequencing of model rhizobia: *Mesorhizobium loti* (Kaneko et al., 2000), *S. meliloti* (Barnett et al., 2001; Capela et al., 2001; Galibert et al., 2001), *B. japonicum* (Kaneko et al., 2002), *Rhizobium etli* (Gonzalez et al., 2006), and *R. leguminosarum* (Young et al., 2006), a very large number of rhizobial genomes have been sequenced or are in the process of being sequenced (as many as 263 complete or ongoing genome sequence projects in GOLD, the Genomes OnLine Database – http://www.genomesonline.org, as of April 30, 2013, and over 300 if members of the related *Agrobacterium* genus are included). Although data are still quite recent, several general conclusions on the structure and organization of rhizobia emerge. In general, the rhizobia contain very large genomes, up to *circa* 9 Mb in the case of *Bradyrhizobium* sp. These are the largest proteobacterial genomes, and are among the largest prokaryotic genomes after those of other soil bacteria, such as members of the myxobacteria and actinobacteria (10–12 Mb). The symbiotic region of *B. japonicum*, however, clusters within a genomic island of just *circa* 410 kb (Gottfert et al., 2001). The genomes of other relevant genera (*Rhizobium, Sinorhizobium*) are also very large (*circa* 7 Mb). The occurrence of very large genomes in soil bacteria has been interpreted as an adaptation to this habitat, a complex, hostile, and changing environment that demands the large metabolic and behavioral plasticity that can be provided by a large gene-encoding capacity. Contrary to members of the genus *Bradyrhizobium*, both *Sinorhizobium* and *Rhizobium* present a multipartite genome, harboring several large plasmids, some of which resemble chromosomes ("chromids" (Harrison et al., 2010; see Chapter 26)). On average, 30–40% of the genome in these bacteria is present in the form of plasmids (Galardini et al., 2013; Harrison et al., 2010; Mazur et al., 2011). This characteristic, shared with the *Roseobacter* clade (Petersen et al., 2013), affords a large genomic plasticity, given that many of these plasmids incorporate conjugative systems (Crossman et al., 2008), and liberates these bacteria from the constraints of long replication times associated with a single, very large chromosome.

72.3 METAGENOMICS

Next-generation sequencing approaches can be also applied to the study of the genomic contents of specific environments without the need to isolate or cultivate the organisms therein. This has resulted in unprecedented opportunities to reveal the role and nature of the "hidden majority" in the microbial world and in the development of the discipline of metagenomics (Riesenfeld et al., 2004; National Academy of Sciences, 2007), a term first coined 15 years ago (Handelsman et al., 1998). Since then, metagenomics has revolutionized Environmental Microbiology as an emerging approach that aims to understand the genomic potential of an entire microbial community present in a complex ecosystem (National Academy of Sciences, 2007; Guazzaroni et al., 2009; Morales and Holben, 2010; Simon and Daniel, 2011; de Bruijn, 2011a,b).

Recent technical advances that resulted in cost-effective, massive sequencing technologies and the development of specific bioinformatics tools for the analysis of metagenomic data have increased the number of metagenomic sequencing projects. As of April 30, 2013, the MetaGenomics Rapid Annotation using Subsystem Technology (MG-RAST) Server (http://metagenomics.anl.gov) holds 358 public projects and over 12,000 datasets, totaling over 53 billion sequences (circa 5.5 Tb), with an average metagenome size of 2.3 Gb per dataset.

72.4 SOIL METAGENOMICS AND THE RHIZOBIA

Soil microbial communities have the highest level of prokaryotic diversity, and it is estimated that 1 g of soil can contain up to 10^9 microorganisms (Knietsch et al.,

2003; Delmont et al., 2011b). Metagenomic approaches would appear to be the ideal approximation to study such a complex system. However, even metagenomics is faced with limitations that arise precisely from this complexity. On the one hand, the sheer size of these datasets severely limits our ability to analyze them (Delmont et al., 2011b, 2012; Lombard et al., 2011; Jansson, 2012). On the other hand, soil is an environment that changes rapidly not only temporally but also spatially, even at the microlevel, and its physicochemical properties affect microbial distribution within the soil matrix, which imposes serious technical and methodological problems (Delmont et al., 2011a, 2012; Lombard et al., 2011). Despite these caveats, metagenomics constitutes a powerful approach to obtain information about the nature, composition, and function of microbial communities in soil.

The specificity of the *Rhizobium*–legume symbiosis has traditionally allowed the use of most probable number (MPN) techniques to enumerate soil rhizobia that are able to nodulate trap plants (Brockwell, 1963; Vincent, 1970). In the past years, numerous field studies that include an estimation of symbiotically proficient *Rhizobium* populations have been carried out with different trap plants. For *R. leguminosarum* bv. viciae, representative abundances are as follows (per gram of soil): Eastern France, $0.2{\cdot}10^2$–$1.2{\cdot}10^5$, with pea plants (Louvrier et al., 1996; Depret et al., 2004); Northeastern Germany, 1.4–$8{\cdot}10^4$, with *V. hirsuta* trap plants (Lakzian et al., 2002); Spain, 10^4–10^5, with *Pisum sativum* (our unpublished data); South Australia, 0–$3.2{\cdot}10^4$ (Ballard, 2004) or 0–$7.4{\cdot}10^4$ (Drew et al., 2012) both with *P. sativum* as trap plant. Louvrier and collaborators developed a semiselective medium to isolate *R. leguminosarum* directly from soils (Louvrier et al., 1995). The numbers they obtained in soils from Eastern France were circa 10^4 per gram of soil. Overall, it can be concluded that, although cultivation of the plant host results in an increase in rhizobial soil counts (albeit modest for *R. leguminosarum* (Kucey and Hynes, 1989; Hirsch, 1996)), established soil populations of *R. leguminosarum* are, at most, on the order of 10^4–10^5 per gram of soil. If typical soils are estimated to contain 10^9 bacteria per gram of soil (Knietsch et al., 2003; Delmont et al., 2011b), *R. leguminosarum* would amount to, at most, 0.01% of the total soil microbiota. In practical terms, this implies that even in one of the largest metagenomic datasets (circa 1 Tb), at most 100 Mb would be *R. leguminosarum* DNA. This, barring the crucial problem of how to specifically identify these sequences, would represent at most a 15× coverage of a single *R. leguminosarum* genome and would barely be the representative of the population diversity. Thus, the low natural abundance of rhizobia in soils precludes the use of purely metagenomic methods to study their diversity and demands an alternative approach.

72.5 THE POOL-SEQ APPROACH TO STUDY RHIZOBIAL POPULATIONS

Kofler and collaborators, working with *Drosophila melanogaster* populations, proposed for the first time the Pool-Seq term in 2011 for the next-generation sequencing and analysis of pooled DNA samples from natural populations. It constitutes a cost-effective (and hence feasible), genome-wide approach for comparison of population samples, thus allowing an easy scaling from the limitations of single markers to population genomics (Futschik and Schloetterer, 2010; Kofler et al., 2011a,b).

We reasoned that sequencing pooled DNA samples from *R. leguminosarum* bv. viciae nodule isolates obtained from different legume plant hosts used as rhizobial traps would allow an experimental test of the hypothesis that different plant hosts select specific subpopulations of rhizobia from the available population present in a given soil. We compared two nodule populations (*P. sativum* and *V. faba*) originating from the same agricultural soil. Each subpopulation contained 100 isolates that were grown independently. They were adjusted to the same optical density, strains from the same host were pooled, and genomic DNA from the pooled culture was extracted (Wilson, 2001). Genomic DNA from each pool was sequenced (Illumina Hi-Seq 2000, 180 bp PE libraries, 100 bp reads, 12 Mreads) at BGI (Hong Kong and Shenzhen, China).

In analyzing rhizobial Pool-Seq data, two specific considerations must be taken into account.

First, plant-specific subpopulations derive from the same unselected, resident soil population, whose genomic composition is, by definition, unknown, since their low numbers preclude any unselected genomic analysis. It is likely that this resident population contains both major and minor genomic types, resulting both from the soil's edapho-climatic properties and from its agricultural history. Thus, specific selection by the legume host will operate – if it does – on this original composition which, although distorted by the plant effect, will still be present in the plant-specific isolates.

Second, the large size and the multipartite composition of the *R. leguminosarum* genome (see earlier) favor both an open pan-genome and a large nonconserved (auxiliary or accessory) genome (Gonzalez et al., 2010; Lozano et al., 2010; Bailly et al., 2011). With *R. leguminosarum* bv. viciae, it has been estimated that 20–30% of the genes are strain specific (our unpublished results). This suggests that plant host selection of specific rhizobial genotypes may implicate specific genes or groups of genes (e.g., transport and metabolism of substrates). However, identification of these genes from Pool-Seq data is technically complex, since any DNA assembly will result, necessarily, in the formation of chimeras with no biological meaning.

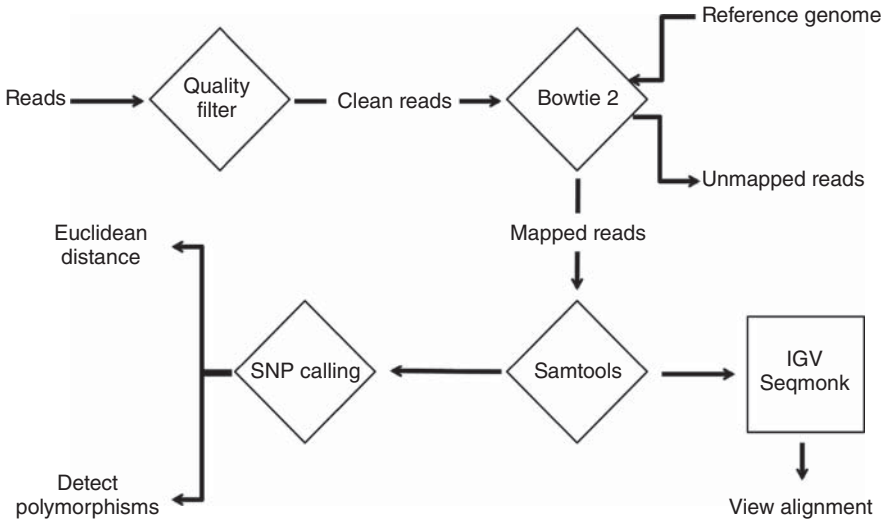

Figure 72.1 Data flow chart of the Pool-Seq DNA dataset analysis. Details are presented in the text.

With these limitations in mind, we decided to restrict the Pool-Seq comparative data analysis to conserved genes, and reads for those genes were identified following recruitment by a reference genome, which in our case was that of *R. leguminosarum* bv. viciae 3841 (Young et al., 2006). For this purpose, a data analysis pipeline was designed and implemented (Fig. 72.1). In the pipeline, after alignment of reads to the reference genome, both coverage and single nucleotide polymorphism (SNP) analysis are performed and compared between subpopulations. The rationale and operation of the pipeline are described later.

72.6 OPERATION OF THE POOL-SEQ DATA ANALYSIS PIPELINE

72.6.1 Data Cleaning

After receipt of sequence data, they must be quality-filtered. A large number of freely available tools exist, but we favor Trimmomatic (Lohse et al., 2012). It contains simple and efficient algorithms to filter by Phred-quality values.

72.6.2 Data Alignment against a Reference Genome

Many different algorithms are available to align clean reads against a reference genome, such as BLAST (Altschul et al., 1990), Maq (Li et al., 2008), Soap2 (Li et al., 2009b), and BWA (Li and Durbin, 2010). We chose Bowtie2 (Langmead and Salzberg, 2012) because of its speed and accuracy. Bowtie was designed for very fast alignment of a high number of reads against a reference genome. The last version allows the alignment in the presence of gaps, which is very useful for rhizobia because their genomes present a large number of insertion sequences (ISs) (Freiberg et al., 1997; Hernández-Lucas et al., 2006; Lozano et al., 2010). ISs are especially abundant in chromosomal islands and in plasmids, including symbiotic plasmids. Reads were aligned against strain 3841 as the *R. leguminosarum* bv. *viciae* reference genome using the "very-sensitive" option among the different default alignment methods that Bowtie2 offers. The complete alignment takes a just few hours on a standard desktop computer with 12 Gb RAM.

72.6.3 Coverage Analysis

In order to study coverage and read recruitment, Bowtie2 standard output must be first transformed with SAMtools (Li et al., 2009a) and can be visualized with any of a number of visualizers. We have used both Seqmonk (http://www.bioinformatics.babraham.ac.uk/projects/seqmonk/) and IGV (Robinson et al., 2011). Both visualizers allow an easy detection of genes or genome areas in the reference genome that are present or absent in the dataset. Seqmonk is quite useful to compare different populations, since it was primarily designed to analyze RNA-Seq data, and thus it allows statistical evaluations. IGV, on the other hand, is able to display coverage along the different replicons, as well as SNPs for each position in the reference genome. Quite usefully, it allows the definition of SNP frequency thresholds. Figure 72.2 shows graphical displays for coverage analysis of the different replicons if it is reference strain 3841.

72.6.4 SNP Calling

In order to compare SNP distribution, the SAMtools package is used. One of the scripts in the package, *mpileup*, was designed to compare different alignments carried out against the same reference genome. Its output is a tab-delimited file, where positions in the reference genome correspond to consecutive lines, and each line contains the corresponding

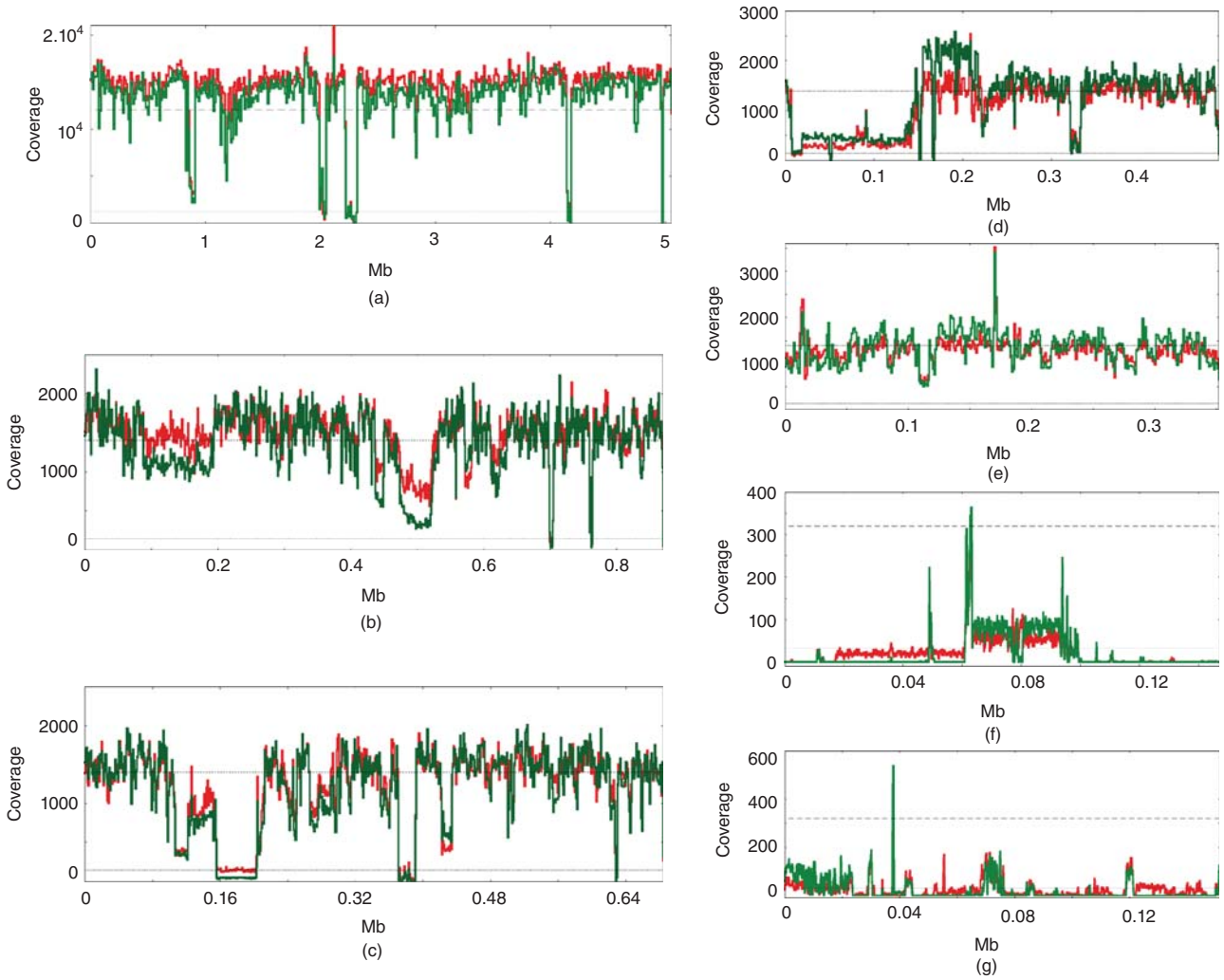

Figure 72.2 Coverage analysis of the *P. sativum* and *V. faba* Pool-Seq datasets after Bowtie2 alignment against the *R. leguminosarum* bv. viciae 3841 reference genome. (a) Chromosome; (b) plasmid pRL12; (c) plasmid pRL11; (d) plasmid pRL10 (the symbiotic plasmid); (e) plasmid pRL9; (f) plasmid pRL8; and (g) plasmid pRL7. Read coverage was plotted in red for *P. sativum* and in green for *V. faba*, along 10,000 bp (for panel a), 1000 bp (for panels b–d), and 100 bp (for panels e–f) windows.

nucleotides of the reads that are mapped in each population to that position. Several scripts are available that can read an *mpileup* file and call for variants. Most of them are designed for diploid organisms, but can also be used with haploid organisms. We chose VarScan (Koboldt et al., 2012) for SNP calling. The program reports only those positions where a variant is present and applies a Bayesian algorithm, assigning a *p*-value to all SNP calls. Figures 72.3 and 72.4 show graphical displays for SNP analysis.

72.6.5 Population Comparisons

With this approach, SNP patterns within genes or gene clusters are easily visualized for genes or gene clusters of interest. Results presented in Figures 72.3 and 72.4 show that a legume host plant-specific pattern of SNP distribution exists, at least for the randomly chosen marker gene *atpD* and gene cluster *nifHDK*, respectively, thus confirming that in the *R. leguminosarum* bv. viciae symbiotic system, the plant host selects specific genotypes from those available in the soil.

It is of course possible to extend this analysis to a large number of genes and gene clusters and to a larger number of legume hosts (such as *Lens*, *Lathyrus*, and vetches, such as *V.* sativa), and also globally to the whole conserved genome and to all the available polymorphic sites. In order to carry out this analysis, results from SNP calling are used to calculate Euclidean distances among populations (with the help of any statistical package such SPSS v.20). These Euclidean distances can also be graphically plotted in a 2D space (data not shown).

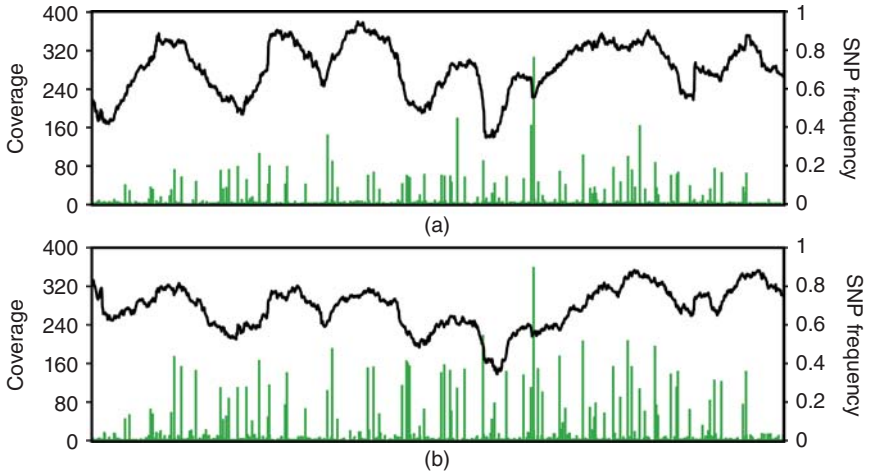

Figure 72.3 Coverage and SNP frequency analysis of Pool-Seq datasets against the *R. leguminosarum* bv. viciae 3841 *atpD* gene. (a) *P. sativum* dataset and (b) *V. faba* dataset.

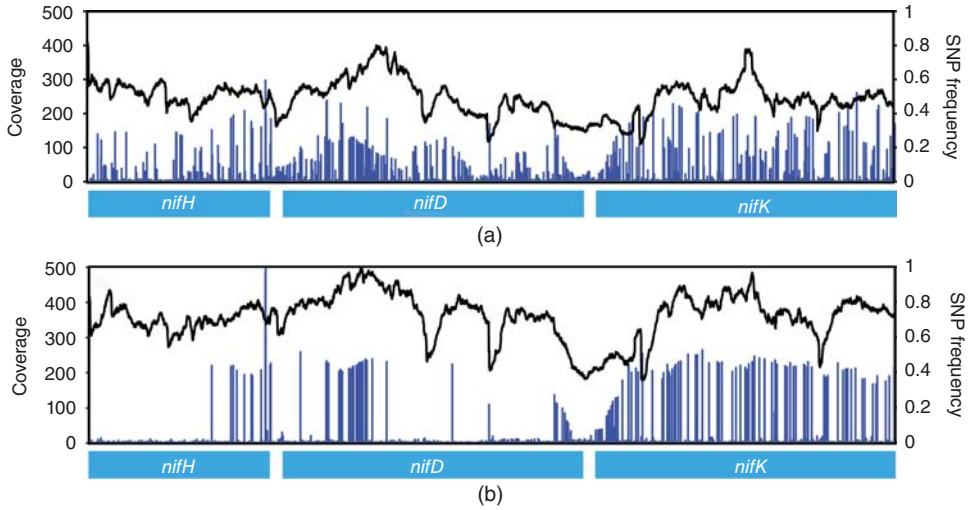

Figure 72.4 Coverage and SNP frequency analysis of Pool-Seq datasets against the *R. leguminosarum* bv. viciae 3841 *nifHDK* gene cluster. (a) *P. sativum* dataset and (b) *V. faba* dataset.

72.7 A CAUTIONARY NOTE ON METAGENOMIC ANALYSIS OF POOL-SEQ DATA

Pool-Seq datasets do not, in principle, differ much from metagenomic datasets, except for their very low complexity and very high coverage of specific DNA regions. *R. leguminosarum* datasets were uploaded to the MG-RAST server (Meyer et al., 2008) and analyzed. A phylogenetic 16S analysis was first carried out with the different databases available in MG-RAST. Since all 100 strains in the original Pool-Seq sample were isolated from symbiotically active *P. sativum* or *V. faba* nodules, the analysis was expected to identify *R. leguminosarum* exclusively. However, results from the MG-RAST pipeline suggest a population far more complex than expected (Fig. 72.5). Interpretation of these unexpected results is currently underway.

72.8 DISCUSSION: PROS AND CONS OF POOL-SEQ ANALYSIS FOR THE STUDY OF RHIZOBIAL POPULATIONS

Since rhizobial populations are a minor fraction of the soil microbiota, any attempt to approach the genomic structure of such populations will necessarily require a preliminary enrichment through the use of legume host trap plants, thus potentially introducing a host-mediated bias. Our work discussed here provides genomic evidence that such a bias does exist, and that specific hosts select specific genotypes from the available variability present in the soil. This evidence has been obtained by next-generation sequencing of DNA samples pooled from a large number (100) of root nodule isolates of *R. leguminosarum* bv. viciae from each of two different legume plant hosts, followed by coverage

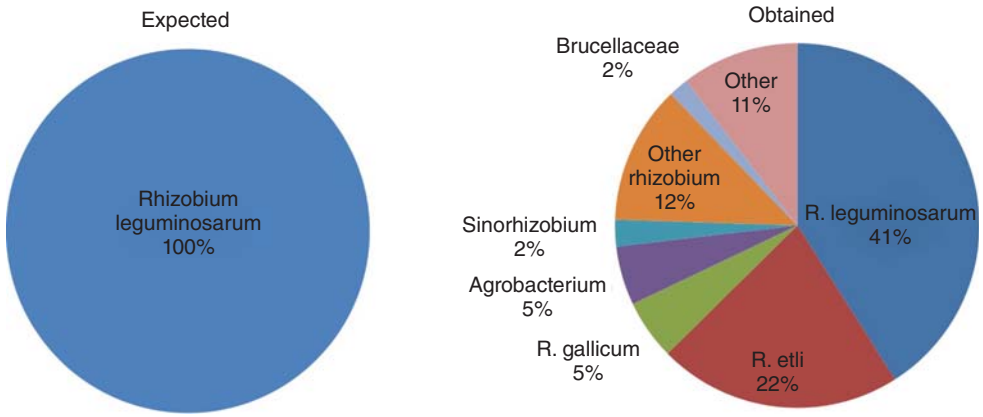

Figure 72.5 Expected and obtained 16S rDNA-based phylogenetic distribution from the MG-RAST analysis of the *P. sativum* Pool-Seq dataset.

and SNP analysis of these datasets against the genomic sequence of reference strain *R. leguminosarum* bv. viciae 3841. These analyses have been carried out with a data processing pipeline assembled from freely available tools.

Given unlimited resources for sequencing and data analysis, it is clear that individual genome sequencing of isolates followed by assembly and multiple genome comparisons represents a more powerful tool than Pool-Seq. However, such a situation is unlikely to occur, and the advantages and disadvantages of Pool-Seq must be evaluated for each project. In 2010, when this project was designed, it was not cost-effective to individually sequence and assemble 200 rhizobial strains. Even with today's higher capacity and lower costs, the pooled DNA approach would allow for a higher sequencing depth, and thus for a potentially better description of the populations. On the other hand, the Pool-Seq approach suffers from important drawbacks for this type of analysis in rhizobia. First, since the plant-specific genotype enrichment will necessarily reflect the original structure of the rhizobial population in the soil, and this can vary from soil to soil, the analysis should be repeated with different types of soil. And second, and more important, the impossibility to assemble reads without the generation of chimeras makes it very difficult to identify specific genes that are not present in the reference genome but that may be specifically enriched in plant-selected subpopulations. These genes are important because they can provide not only specific host-linked markers but also evidence for the structural or functional nature of the phenotypes selected by the plant. Two complementary approaches come to mind in order to address this limitation of Pool-Seq analysis. First, the complexity of the plant-enriched subpopulations could be reduced by any number of typing methods, for instance random amplified polymorphic DNA (RAPD) analysis (Moschetti et al., 2005), making this reduced number of isolates amenable to direct genome sequencing and assembly. Second, our Pool-Seq pipeline for coverage and SNP data analysis could be repeated with different *R. leguminosarum* reference genomes in order to incorporate coverage and SNP analyses for genes that were absent from the original reference genome. Both strategies are currently being followed in our laboratory to complement our analyses.

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Chapter 73

Contribution of the RNA Chaperone Hfq to Environmental Fitness and Symbiosis in *Sinorhizobium meliloti*

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73.1 INTRODUCTION

The Hfq protein was discovered more than 40 years ago in *Escherichia coli* as a factor required for the replication of the RNA phage Qβ (Franze de Fernández et al., 1968). The rapidly growing genomic database reveals that nearly half of the sequenced bacterial species and a few archea encode a recognizable homolog of this protein, which is usually highly represented in the proteome repertoire. Genetic, biochemical, and structural data evidence a quaternary arrangement of Hfq into a hexameric toroid structurally related to the eukaryotic LSm family of RNA-binding proteins (Brennan and Link, 2007).

Deletion of the chromosomal *hfq* gene was early observed to impair multiple responses to stressful environmental conditions in *E. coli* (Tsui et al., 1994). Subsequently, similar reverse genetic approaches have confirmed the pleiotropy of the *hfq* mutation in a number of phylogenetically distant bacterial species representing diverse lifestyles (Sobrero and Valverde, 2012 and references therein). In animal pathogens (e.g., enterobacteria or *Brucella* species), the absence of Hfq has been shown to attenuate motility, secretion of virulence factors, host invasion, or intracellular survival of bacteria (Robertson and Roop, 1999; Sittka et al., 2007). These findings predict a universal role of Hfq in the establishment and maintenance of chronic intracellular residences within eukaryotic hosts, such as that involving root nodule colonization by nitrogen-fixing endosymbiotic bacteria.

Extensive work on model enterobacteria has revealed that loss of Hfq results in the deregulation of large arrays of genes and operons encoding widely diverse cellular functions that include transport, metabolism, and tolerance to a variety of stresses (Sittka et al., 2007, 2008). This emerging role of Hfq as a global regulator of gene expression mostly relies on its RNA-binding capacity, which renders this protein as a crucial node in bacterial RNA transactions (Vogel and Luisi, 2011). Remarkably, Hfq is the major binding protein for *trans*-acting small noncoding RNAs (sRNAs), which constitute the largest and most intensively investigated class of regulatory untranslated RNA molecules recently identified in bacteria (Storz et al., 2011). Upon binding, Hfq promotes the short, discontinuous, and imperfect antisense interaction of the *trans*-sRNAs with their mRNA targets, thereby modulating translation and/or turnover rates of the messages. The homo-hexameric Hfq

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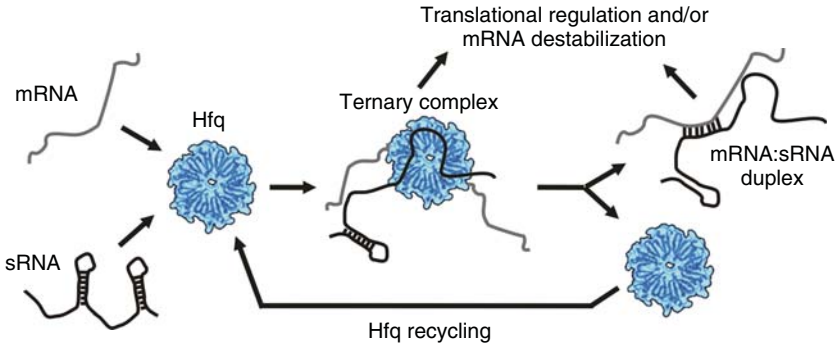

Figure 73.1 Hfq facilitates annealing between an sRNA and its cognate mRNA. The Hfq hexamer can bind different RNA molecules in either of its positively charged surfaces. RNA binding may result in secondary structure changes that promote base-pairing recognition and duplex formation due to proximity in the ternary complex. The bound sRNA may block (or even facilitate) ribosome access to the mRNA; alternatively, Hfq-recruited RNase may irreversibly downregulate mRNA expression (for further details, see Vogel and Luisi (2011)).

ring exposes two different positively charged surfaces (i.e., the proximal and distal faces), which constitute alternative binding sites that can discriminate between RNA molecules (Fig. 73.1). Studies on *Staphylococcus aureus* and *E. coli* Hfq have revealed that the proximal face has a preference for uridine-rich RNA stretches, which seem to be accommodated around the pore in a constricted conformation that is stabilized by water molecules (Schumacher et al., 2002; Sauer and Weichenrieder, 2011). As most sRNAs have typical bacterial Rho-independent terminators that usually contain a poly-U 3′-terminus, Hfq can interact with the terminators and influence sRNA stability (Vogel and Luisi, 2011). In contrast, the *E. coli* Hfq distal face presents an RNA-binding motif with preference for adenine-rich RNA segments. In this case, the RNA molecule is accommodated in a circular conformation along the distal surface. Hence, each Hfq ring is able to simultaneously bind two different RNA molecules or a single molecule bridging both faces around the oligomer rim (Fig. 73.1). If an sRNA binds on one face and a cognate target mRNA does so on the other face, this ternary complex will lead to productive RNA duplex formation (Wang et al., 2013). In addition, Hfq offers a scaffold for the interaction with several other proteins (Sobrero and Valverde, 2012). The more relevant partner of Hfq is the major bacterial ribonuclease, RNase E, which engages in the formation of an atypical degradosome (Morita et al., 2005). This silencing complex ensures the efficient modulation of the riboregulatory networks (Aiba, 2007).

Posttranscriptional regulation of gene expression mediated by *trans*-sRNAs contributes to fine-tune bacterial processes as diverse as sugar and amino acid transport and metabolism, iron and envelope homeostasis, general responses to abiotic stress, quorum sensing, or virulence (Storz et al., 2011). Even though Hfq can directly influence mRNA translation and stability, most of the Hfq-dependent genes are expected to be regulated by the concerted activity of this protein and its interacting *trans*-sRNAs.

In the order Rhizobiales, Hfq was first identified in *Azorhizobium caulinodans* as a protein factor required for the proper translation of the master regulator *nifA* (Kaminski et al., 1994); this observation lead to the designation NrfA (*nifA* regulatory factor) of the protein. The requirement of Hfq for *nifA* expression seems to be conserved in other α-proteobacteria, such as *Rhizobium leguminosarum* bv. *viciae* (Zhang and Hong, 2009) and *Rhodobacter capsulatus* (Drepper et al., 2002). Indirect evidence for Hfq-dependent regulation of genes involved in nitrogen metabolism has been revealed in *R. leguminosarum* bv. *viciae*, as spontaneous *hfq* point mutations arise as natural suppressors of a mutation in *gltB* (encoding the large glutamine oxoglutarate amidotransferase (GOGAT) subunit) that inhibits amino acid uptake through the Aap and Bra transport systems (Mulley et al., 2011).

More than one decade after the initial experiments in *A. caulinodans*, the exploration of the biological function of Hfq in rhizobia has recently been resumed in *Sinorhizobium meliloti*, upon detection of many sRNA transcripts in different strains (del Val et al., 2007; Ulvé et al., 2007; Valverde et al., 2008; Schlüter et al., 2010, 2013). Altogether, the identification of sRNAs and of an *hfq* allele in *S. meliloti* strongly suggested that Hfq-dependent riboregulation impacts physiology of this bacterium under both free-living and symbiotic conditions. In this chapter, we summarize our current knowledge on the biology of Hfq in *S. meliloti*.

73.2 THE *S. meliloti* hfq PROTEIN

In all sequenced *S. meliloti* strains, the *hfq* allele is invariably located in the chromosomal replicon, downstream of the gene encoding a putative D-alanine aminotransferase, and upstream of *hflX*, which encodes a putative guanosine triphosphatase (GTPase) of yet unknown function (Fig. 73.2a). Intriguingly, a number of genes involved in transcriptional regulation of nitrogen uptake and metabolism (i.e., *ntrBC*, *ntrYX*) are physically linked upstream of *hfq*, a feature that is also shared by other rhizobia and related α-proteobacteria. The *hfq-hflX* tandem is conserved in α-, β-, and γ-proteobacteria (Sobrero and Valverde, 2012), suggesting some degree of functional relationship. In fact,

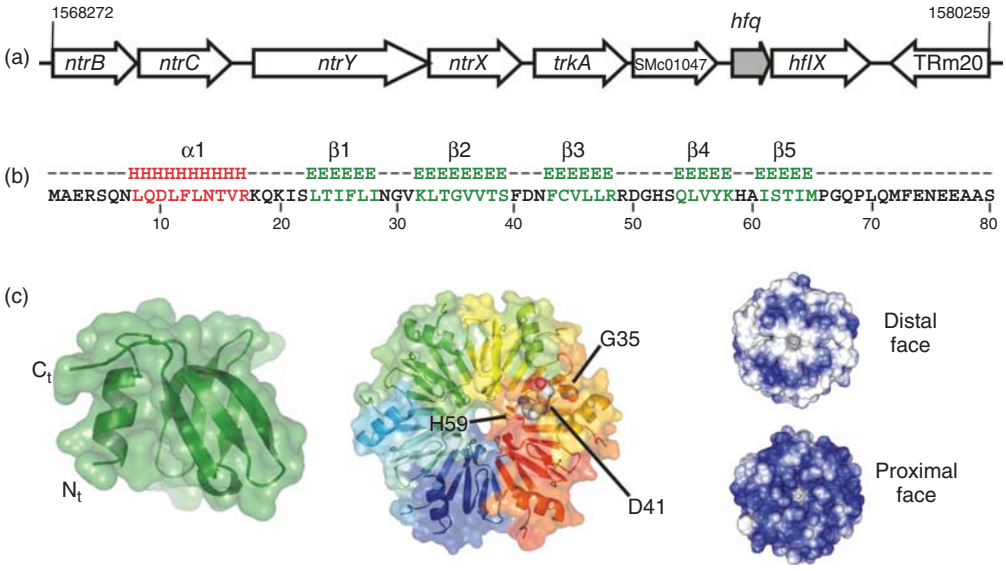

Figure 73.2 Sequence and structural properties of *S. meliloti* Hfq. (a) Genetic context of the 12-kb region encompassing *hfq* in the chromosome of *S. meliloti* strain Rm1021. (b) Amino acid sequence and predicted secondary structure elements of Hfq (predicted α-helical and β-stranded regions are indicated above the sequence). (c) Predicted ribbon model of the Hfq monomer using the *E. coli* Hfq polypeptide (PDB 1hk9) (protein data bank) as a template (left). The spatial location of critical conserved residues important for Hfq function is indicated in the hexamer model (middle). Consistent with their RNA-binding properties, the two faces of the hexameric Hfq ring have different predicted positive net charge (highlighted in blue in the right panel).

hflX is cotranscribed with *hfq* in *S. meliloti* (Sobrero and Valverde, 2011).

The *S. meliloti* Hfq monomer has 80 residues and a calculated molecular weight of 9 kDa (Fig. 73.2b). The sequence is 47% identical to that of *E. coli*, although the identity is concentrated in the first 70 residues, provided that the *E. coli* monomer is slightly larger (102 residues). The *S. meliloti* Hfq polypeptide is predicted to fold into an N-terminal α-helix followed by a tandem of five β-strands (Fig. 73.2b), a topology that has been confirmed by the crystallographic studies of *E. coli* Hfq. The significant primary and secondary structure homologies found among *S. meliloti* and *E. coli* monomers strongly suggest that *S. meliloti* Hfq oligomerizes into a 54-kDa ring-shaped hexamer with two positively charged surfaces (Fig. 73.2c). Key residues for stabilization of the *E. coli* Hfq fold are conserved in the primary sequence of *S. meliloti* Hfq (e.g., G35, D41, and H59) and are positioned in equivalent locations of the hexameric model (Fig. 73.2c), suggesting that they are relevant for the native structure of *S. meliloti* Hfq as well. In fact, an Hfq variant carrying a G35V substitution failed to complement an *hfq* deletion in strain 1021 (Barra-Bily et al., 2010a).

In *S. meliloti*, *hfq* expression is driven by a conserved and prototypical rhizobial σ^{70} promoter (Sobrero and Valverde, 2011), both in free-living and symbiotic states (Barra-Bily et al., 2010a; Sobrero and Valverde, 2011). A remarkable feature of *hfq* expression is that its translational rate remains constant along the growth curve under different nutritional conditions, which points to a tight control of Hfq protein level (Sobrero and Valverde, 2011). This is consistent with the notion derived from enterobacterial models that this RNA chaperone is a limiting factor of riboregulatory networks, with a rather stable number of molecules per cell; in this way, mRNA–sRNA regulatory outputs would be more sensitive to fluctuations in sRNA levels (Adamson and Lim, 2011; Moon and Gottesman, 2011). Interestingly, this precise control of Hfq protein levels relies on an autoregulatory loop that involves translational repression of its own mRNA. *E. coli* Hfq binds to its own mRNA leader, thus repressing *hfq* translation by competing with the translation machinery for the ribosome-binding site (RBS) (Vecerek et al., 2005). In *S. meliloti*, Hfq controls the expression of an *hfq-lacZ* translational fusion, and this process only requires the presence of the Hfq protein (Sobrero and Valverde, 2011). Whether sRNAs targeting the *hfq* mRNA leader of *E. coli* and *S. meliloti* contribute *in vivo* to *hfq* translational autoregulation is not clear. Furthermore, indirect evidence of *hfq* autoregulation has also been obtained in another α-proteobacterium, as the *R. sphaeroides hfq* mRNA was detected in the RNA pool that co-immunoprecipitated (CoIP) with Hfq (Berghoff et al., 2011). The operation of an Hfq autoregulatory loop in different bacterial lineages emphasizes the relevance of keeping Hfq protein concentration locked within a limited range.

73.3 DELETION OF *hfq* RESULTS INTO A PLEIOTROPIC FREE-LIVING AND SYMBIOTIC PHENOTYPE

Several lines of experimental evidence indicate that Hfq has a pivotal role in maintaining cellular homeostasis and fitness of *S. meliloti*. A series of contemporary studies have revealed the phenotype of *hfq* mutants derived from the closely related *S. meliloti* strains Rm1021 and Rm2011 (Barra-Bily et al., 2010a; Gao et al., 2010; Torres-Quesada et al., 2010; Sobrero and Valverde, 2011). Both strains, which arose from the same streptomycin-resistant isolate SU47, have been independently domesticated and have accumulated genotypical and phenotypical variations (Sallet et al., 2013), particularly in their response to phosphate limitation and in their interaction with the host plant (Wais et al., 2002; Krol and Becker, 2004). However, regardless of the mutated strain, the lack of *hfq* results in shared pleiotropic phenotypes, namely (a) a reduced specific growth rate, longer lag phase, and lower biomass yield in planktonic growth in complex and nutrient-rich medium; (b) the motility/chemotactic behavior on semisolid medium is severely affected; and (c) increased sensitivity to heat shock, oxidative stress, and high pH.

Probably as a consequence of these series of alterations, the *S. meliloti* wild-type strain Rm1021 strongly outcompetes its *hfq*-mutant derivative for nodule occupancy in coinoculation experiments of alfalfa plants grown hydroponically (Torres-Quesada et al., 2010). Differential traits of the *S. meliloti hfq* mutants also become evident when examining the interaction with the host plant in single inoculation experiments. Both Barra-Bily et al. (2010a) and Gao et al. (2010) describe a strong Nod^+ Fix^- phenotype for the *hfq* mutant derived from strain Rm1021, that is, bacteria are able to induce nodule formation but cannot colonize and fix nitrogen within these organs. Thus, a high proportion of the developed nodules remain white (Fix^-). Reisolation and reinoculation of rhizobia from the fewer pink and elongated nodules did not result in a higher proportion of Fix^+ nodules (Barra-Bily et al., 2010a), arguing against plant selection of *hfq* suppressing mutations. The infective defect could be a consequence of the higher sensitivity of the *hfq* mutants to the oxidative stress generated by the plant cells during root hair penetration and nodule cell invasion. This oxidative burst constitutes an important baseline defense mechanism against different invading bacteria and fungi (Santos et al., 2001). Another study reported a moderate Nod^+ Fix^- phenotype of a strain Rm1021 *hfq* mutant, although in this case the histology of the handicapped nodules suggested an impaired ability of bacteroids to survive within the nodule cells (Torres-Quesada et al., 2010). This phenotype further supports a common role of Hfq in the chronic intracellular infection of eukaryotic hosts by bacteria. Finally, an *hfq*-mutant derivative from strain Rm2011 displays a Nod + Fix + phenotype upon inoculation of lucerne seedlings, but there is a marked delay in the appearance of the first nodule with the consequent shift in the distribution of nodules toward younger portions of the root system (Sobrero and Valverde, 2011). Thus, nodule maturation is delayed and results in a retardation of nitrogen supply for plant growth. The differential symbiotic performance of the *hfq* mutants could be attributed to the host plant species (*M. sativa* (Barra-Bily et al., 2010a; Torres-Quesada et al., 2010; Sobrero and Valverde, 2011) or *M. truncatula* (Gao et al., 2010)) or to the lucerne variety (cv. Iraquois (Barra-Bily et al., 2010a), cv. Aragón (Torres-Quesada et al., 2010), or cv. Key (Sobrero and Valverde, 2011)), which may vary in the amplitude of the oxidative burst triggered by the initiation of the infection process (Santos et al., 2001). In summary, the lack of Hfq does not compromise Nod factor signaling in *S. meliloti* but impacts on intermediate and late symbiotic stages.

73.4 A LARGE REGULON EXPLAINS THE PHENOTYPIC PLEIOTROPY OF THE *S. meliloti hfq* MUTANTS

To identify the genes that are directly or indirectly regulated by Hfq, the proteome and transcriptome of *hfq* mutants from strains Rm1021 and Rm2011 grown either in rich or defined minimal media have been explored by 2D-PAGE (Barra-Bily et al., 2010b; Torres-Quesada et al., 2010), quantitative proteomics (Sobrero et al., 2012), or microarray hybridization experiments (Gao et al., 2010; Torres-Quesada et al., 2010). The compilation of the mRNAs/proteins that show differential abundance between the mutants and the parent strains in these studies reveals a large Hfq regulon consisting of 921 genes (306 upregulated, 615 downregulated in the mutants), which represents 15% of the ORFs (Open Reading Frames) annotated in the *S. meliloti* genome (Fig. 73.3). Almost half of these genes putatively encode components of either ATP-binding cassette (ABC) transport systems (24%) or metabolic proteins (22%), whereas 31% have no predictable function. The remaining deregulated genes (22%) specify diverse cellular functions that include motility, chemotaxis, regulation of transcription, or nitrogen fixation. Expression of *S. meliloti* genes related to DNA and protein synthesis is largely unaffected by the lack of Hfq, supporting a global role of this protein in the fine-tuning of the cell homeostasis rather than a housekeeping function, as anticipated by the full viability of the *hfq* mutants.

The majority of ABC transporters that are downregulated in the absence of Hfq are predicted to be involved in the uptake of carbon substrates (e.g., ribose, fructose, *myo*-inositol, or α-glucosides). Conversely, genes encoding ABC systems for the uptake of diverse nitrogen compounds

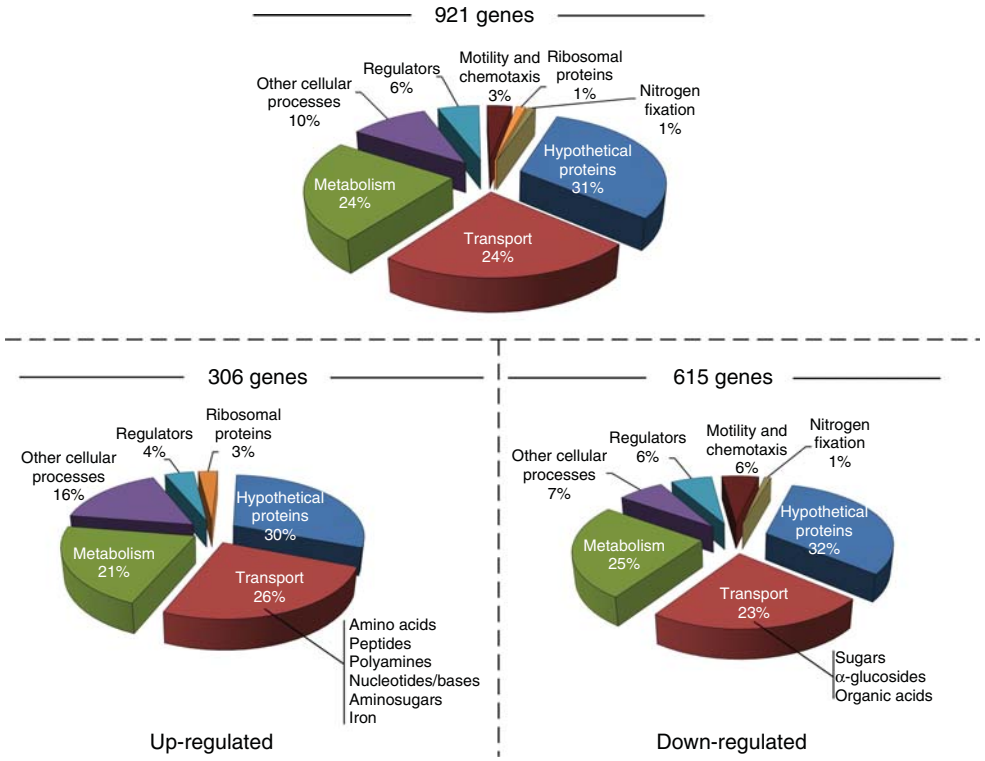

Figure 73.3 The *S. meliloti* Hfq regulon. Functional distribution of Hfq-regulated genes as inferred from transcriptome and proteome profiling of different *hfq* mutants.

(i.e., amino acids, peptides, polyamines, aminosugars, or nucleotides) are massively upregulated in the *hfq* mutants (Fig. 73.3). This observation correlates with an increased sensitivity of the mutants to bialaphos, sodium glufosinate, or 5-fluorouracil, which are cytotoxic analogs of tripeptides, L-glutamate, and uracil, respectively (Sobrero et al., 2012).

The predicted reduced efficiency of the *S. meliloti hfq* mutants in the uptake of primary carbon compounds is accompanied by a reciprocal downregulation of several genes involved in the utilization of sugars and, in turn, of those of biosynthetic pathways of macromolecule building blocks (e.g., amino acids) fueled by carbon catabolism (Torres-Quesada et al., 2010). Consistent with these changes, transcriptomic/proteomic alterations suggest a bias of metabolism to the tricarboxylic acid (TCA) cycle and gluconeogenesis as alternative energy-producing pathways in the mutants (Fig. 73.4). This metabolic shift is likely supported by the aforementioned promotion of the amino acid uptake, the upregulation of the gene encoding the enzyme phosphoenol pyruvate carboxykinase (PckA), and the increase in the nitrogen assimilatory activity in the *hfq* mutants (Barra-Bily et al., 2010b; Gao et al., 2010; Sobrero et al. 2012). However, this metabolic reprogramming seems to be not fully coordinated with transport. For example, syntheses of branched-chain amino acid (leucine, isoleucine, and valine, LIV) ABC transporters (i.e., LivHMGFK and AapJQMP) increase in the *hfq* mutants, but more than a dozen genes encoding LIV catabolic enzymes are downregulated (Gao et al., 2010). Therefore, the growth defects of the *S. meliloti hfq* mutants in unstressed cultures are likely a consequence of these series of specific alterations in the uptake of nutrients and metabolism.

The abundance of a set of proteins involved in the transport (e.g., FbpA and SMc01605) and storage (e.g., bacterioferritin or Bfr) of iron increases in *S. meliloti* Rm2011 lacking Hfq, thus contributing to elevate the intracellular iron content in the mutant (Sobrero et al., 2012). Nonphysiological high-iron concentrations could lead to oxidative stress due to Fenton chemistry in the presence of reactive oxygen species (ROS) (Cornelis et al., 2011). ROS detoxifying enzymes such as catalases (e.g., KatB or KatC) or superoxide dismutases (e.g., SodB and SodC) have been consistently found to be downregulated in the *S. meliloti hfq* mutants, thus reducing their ability to repair oxidative damage during aerobic growth and legume infection (Barra-Bily et al., 2010b; Sobrero et al., 2012). Several additional stress-related genes have been identified as misregulated in the *S. meliloti hfq* mutants in these studies, despite of being mostly conducted under nonstress conditions. Among those, *S. meliloti* Hfq has been shown to contribute to the optimal expression of *rpoE1*, *rpoE2*, *rpoE3*, and *rpoE4*, which encode extracytoplasmic sigma factors (ECFs) involved in the control of responses to a number of abiotic stresses such as oxidative stress or heat shock (Barra-Bily et al., 2010b; see Chapters 30, 40).

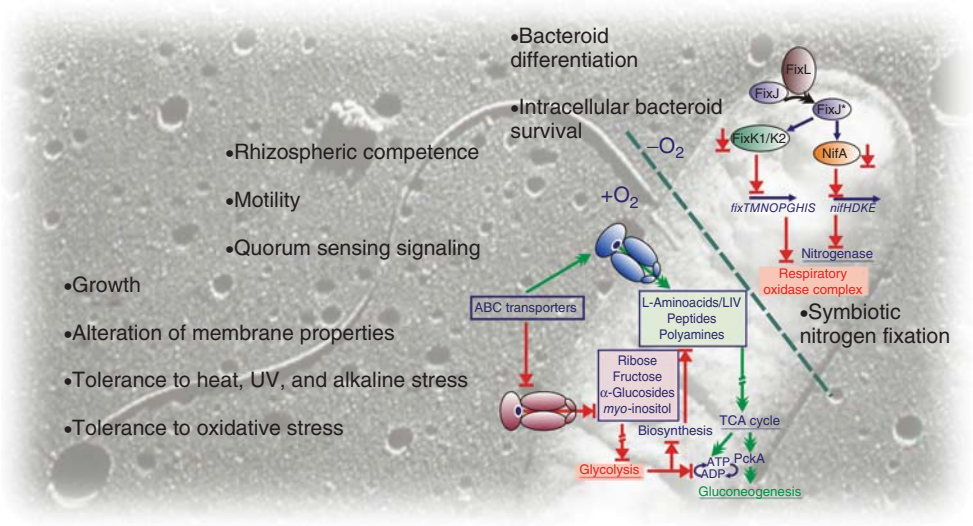

Figure 73.4 Summary of the *S. meliloti* pathways and phenotypes influenced by Hfq. Green double arrowheads and red-blocked arrows denote favored and disadvantaged pathways, respectively, in the absence of Hfq. $+O_2$, aerobic cultures; $-O_2$, microaerobic cultures. See text for details.

Expression of other genes encoding proteins contributing to heat shock recovery (e.g., the chaperones GroES1 and ClpP2) or alkaline tolerance (SupAB) has also been shown to be drastically reduced in a *S. meliloti* Rm1021 *hfq* mutant (Barra-Bily et al., 2010b; Gao et al., 2010).

Several changes in the pattern of gene expression could explain the reduced nodulation competitiveness of the *S. meliloti hfq* mutants (Torres-Quesada et al., 2010). Root exudates and decaying plant matter are likely important sources of carbon compounds such as α-glucosides (trehalose, sucrose, and maltose) or *myo*-inositol for soil-dwelling rhizobia (Fry et al., 2001). The genes encoding functions for the transport and utilization of these substrates are strongly downregulated in the mutants (Gao et al., 2010; Torres-Quesada et al., 2010). On the other hand, lack of Hfq also results in downregulation of most known flagellar and chemotaxis genes and accumulation of the *sinI* mRNA, encoding the acyl homoserine lactone (AHL) synthase, thus leading to defects in swimming/swarming motility and alteration of the quorum-sensing signal profile (Barra-Bily et al., 2010a, Gao et al., 2010; see Chapter 37). These deficiencies would compromise to a different extent that the overall responses of rhizospheric bacteria to symbiotic plant signal.

Contrasting with soil oligotrophy, legumes provide invading rhizobia with defined and abundant energy sources such as dicarboxylic acids, the catabolism of which supports the energy demands of symbiotic nitrogen fixation by the nitrogenase complex (Prell and Poole, 2006). Nonetheless, it has been also reported that transport of plant-derived LIV through the bacterial Aap and Liv(Bra) systems is required for *R. leguminosarum* bv. *viciae* bacteroid development and persistence within pea nodules (Prell et al., 2009). Such a symbiotic function of LIV uptake has not been demonstrated in *S. meliloti* (Prell et al., 2010). However, the substrate specificity of *S. meliloti* LivK and AapJ periplasmic solute-binding proteins is predicted to be broad (Prell et al., 2010; see Chapter 34). Therefore, it cannot be ruled out that the misregulation of Liv/Aap or other ABC transporters involved in the uptake of yet unidentified alternative nutrient sources for *S. meliloti* during infection and bacteroid differentiation would contribute, at least partially, to the endosymbiotic phenotype of the *hfq* mutants.

73.5 hfq CONTRIBUTES TO THE CONTROL OF SYMBIOTIC NITROGEN FIXATION

NifA/FixK-dependent transcription of the nitrogen-fixation genes (*nif/fix*) is oxygen-regulated in legume nodules and requires microoxic conditions in cultured free-living bacteria. Nonetheless, it is known that a moderate decrease in the ambient oxygen concentration in the gas phase of a culture is sufficient to trigger FixLJ-mediated activation of the FixK regulator. However, *nifA* expression demands more stringent microaerobic conditions. The transcriptomic profiling of the *S. meliloti* Rm1021 *hfq* deletion mutant revealed downregulation of mRNAs corresponding to the two copies of *fixK* and to their downstream-dependent genes, which encode the protein components of the respiratory chain associated with the nitrogenase complex (*fixN1*, *fixQ1*, *fixP1*, *fixG*, *fixQ2*, and *fixM*) (Torres-Quesada et al., 2010) (Fig. 73.4). In contrast, differential accumulation of *nifA* was not observed in either of the transcriptomic analyses. Further independent reverse transcription polymerase chain reaction (RT-PCR) experiments on RNA from cultured bacteria subjected to

microaerobiosis confirmed that full expression of the *S. meliloti fixK* and *nifA* genes requires Hfq (Barra-Bily et al., 2010a; Torres-Quesada et al., 2010). Downregulation of *nifA* and *fixK* would compromise efficiency of nitrogenase synthesis and nitrogen fixation, thus providing a further explanation to the negative effects of the lack of Hfq on late stages of the symbiotic interaction of *S. meliloti* with its legume host.

Hfq-mediated posttranscriptional regulation of *nifA* expression in *R. leguminosarum* bv. *viciae* has been shown to involve binding to and cleavage of the 5′ untranslated region (UTR) of the mRNA upon RNase E recruitment (Zhang and Hong, 2009). This Hfq-dependent RNase E cleavage enables translation of the *nifA* message, which is otherwise impaired by an inhibitory structure of the 5′-UTR that is predicted to occlude the RBS. RT-PCR data suggest that Hfq contributes to stabilize the *S. meliloti nifA* and *fixK* mRNAs. However, the mechanisms involved in the positive regulation of these *S. meliloti* genes by Hfq remain to be explored.

73.6 hfq AND RIBOREGULATION IN *S. meliloti*

S. meliloti has been shown to express a plethora of small untranslated RNA molecules, including hundreds of *trans*-acting sRNAs, whose function is mostly unknown (del Val et al., 2007; Ulvé et al., 2007; Valverde et al., 2008; Schlüter et al., 2010, 2013). The most relevant information, but also the bottleneck, to pinpoint the cellular functions of the *trans*-sRNAs is the identity of their mRNA targets. Cognate partner mRNAs of Hfq-dependent *trans*-sRNAs are usually identified among the mRNA populations differentially expressed in the *hfq* mutants.

To date only two α-proteobacterial *trans*-sRNAs homologous to each other designated AbcR1 and AbcR2 have been functionally characterized, both in the phytopathogen *Agrobacterium tumefaciens* (Wilms et al., 2011) and the mammal pathogen *B. abortus* (Caswell et al., 2012). AbcR1 negatively regulates a handful of ABC transporter mRNAs in these bacteria, including a *livK* homolog. The *S. meliloti* AbcR1 homolog (formerly Smr16 or Smr15C2) was identified in an early computational genome-wide screen to search for sRNA genes in the intergenic regions of this bacterium (del Val et al., 2007). *S. meliloti* AbcR1 is an Hfq-dependent sRNA; it coimmunoprecipitates with a chromosomally encoded epitope-tagged Hfq variant and rapidly decays in the absence of this protein (Fig. 73.5a) (Voss et al., 2009; Torres-Quesada et al., 2010; Torres-Quesada et al., 2013).

Computational predictions identified multiple mRNAs encoding the periplasmic component of ABC transport systems as putative targets of AbcR1, most of which

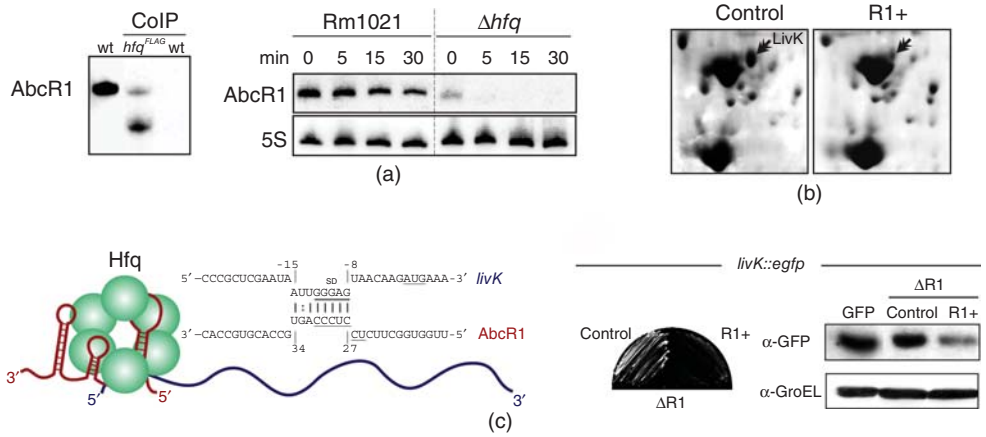

Figure 73.5 Posttranscriptional regulation of *livK* by the *S. meliloti trans*-sRNA AbcR1. (a) AbcR1 is an Hfq-dependent sRNA. Northern blot analysis of RNA species coimmunoprecipitated (CoIP-RNA) with a FLAG-tagged Hfq protein (*hfq*^{FLAG}; left panel) and of AbcR1 decay in Rm1021 and its *hfq* deletion mutant derivative (Δ*hfq*) upon transcription arrest with rifampicin (right panel). Samples were withdrawn prior to or after antibiotic addition at time-points (min) indicated on top of the panel. (b) AbcR1 downregulates LivK. 2D-PAGE analysis of the periplasmic proteome of an Rm1021 AbcR1 deletion mutant (ΔR1; control) and a derivative constitutively expressing AbcR1 from a mid-copy plasmid (R1+). Arrows indicate the LivK spot. (c) AbcR1-mediated translational control of the *livK* mRNA. Left panel: diagram of the predicted antisense interaction between AbcR1 and the Hfq-dependent *livK* mRNA. Numberings denote positions relative to the adenine-uracil-guanine (AUG) start codon of the *livK* mRNA (underlined) and the AbcR1 transcription start site. AbcR1 negatively influences translation and stability of the *livK* mRNA by masking the Shine-Dalgarno sequence (SD) in an Hfq-dependent manner. Right panel: agar plate-based colony fluorescence of the reporter Rm1021 ΔR1 strains cotransformed with a plasmid expressing a translational *livK*::*egfp* fusion and compatible plasmids either empty (control) or expressing AbcR1 (R1+). Western blot analysis of the expression of the LivK::EGFP fusion protein in the same reporter strains is shown to the right. GroEL was probed as protein-loading control.

are Hfq-dependent (Sobrero et al., 2012). Accordingly, periplasmic proteome profiling revealed dowregulation of the LIV-binding protein LivK upon constitutive overexpression of AbcR1 (Fig. 73.5b). Both the *livK* mRNA and its encoded protein have been previously shown to be upregulated in the *S. meliloti hfq* mutants. Scanning of the full-length *livK* mRNA sequence for antisense interactions with AbcR1 revealed a short 8-nt stretch of complementarity between both molecules, which involves the RBS within the target mRNA (Fig. 73.5c). Posttranscriptional AbcR1-mediated control of *livK* has been further confirmed by a double-plasmid reporter assay consisting of the constitutive coexpression of AbcR1 and a translational fusion of the 5′ region of *livK* to enhanced green fluorescent protein (EGFP) from independent compatible plasmids in an *S. meliloti* AbcR1 deletion mutant (Fig. 73.5c). Upon AbcR1 expression, fluorescence of the reporter strain is visibly reduced, which correlates with the downregulation of the LivK::EGFP fusion protein as assessed by Western-blot analysis. Altogether, these data indicate that AbcR1 and Hfq act in concert to inhibit translation of the *livK* mRNA and to accelerate its decay (Torres-Quesada et al., 2013), thus providing an example of the identification of sRNA–mRNA pairs among the cellular Hfq-dependent transcripts.

Preliminary observations indicate that only a subset of *trans*-sRNAs expressed by *S. meliloti* bind Hfq (Torres-Quesada et al., 2010). Therefore, riboregulation may also involve other protein factors in this bacterium. It has been recently reported that a mutation in an eubacterial conserved gene encoding a homolog of the *E. coli* YbeY protein mimicked several phenotypes of the *S. meliloti hfq* mutants (Pandey et al., 2011). YbeY and Hfq do not seem to interact physically in *S. meliloti*. In *E. coli*, YbeY functions as an RNase involved in maturation and quality control of ribosomal rRNA (Jacob et al., 2013). Whether YbeY influences *hfq* expression or function in *S. meliloti*, along with its functional role as an RNase, it remains to be determined.

We are just beginning to uncover the complexity of the noncoding RNome structure in rhizobia. Our current knowledge on Hfq biology provides a solid baseline for the forthcoming systems-level investigation of gene regulation by Hfq-dependent sRNAs in *S. meliloti* and the related α-proteobacteria interacting with eukaryotic hosts.

ACKNOWLEDGMENTS

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Chapter 74

Biodiversity, Symbiotic Efficiency, and Genomics of *Rhizobium tropici* and Related Species

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74.1 INTRODUCTION

Rhizobium tropici is a fascinating, but still little studied, rhizobial species originally isolated from root nodules on common bean (*Phaseolus vulgaris* L.) and species of *Leucaena* in South America (Martínez-Romero et al., 1991). The species is also present in soils of other continents (Africa, Central and North America, Europe, Oceania) and can establish symbiosis with several host species, including legumes indigenous to the Americas and Australia. Interest in the evolution of *R. tropici* results from its close genetic resemblance with agrobacteria, indicating the interesting possibility of discovering the linkage between symbiosis and pathogenicity (e.g., Gomes et al., 2012b). Taxonomy of the species is going through changes, with description and indication of new species, as well as the definition of a "*Rhizobium tropici* group," comprising several species that are genetically related (Ormeño-Orrillo et al., 2012; Ribeiro et al., 2012; Dall'Agnol et al., 2013). In agronomic terms, the high genetic stability of the symbiotic plasmid (pSym), the tolerance to environmental stresses and the high capacity of fixing nitrogen of some elite strains have resulted in increasing use of *R. tropici* strains in inoculants (e.g., Hungria et al., 2000, 2003). In this chapter, we discuss the biodiversity of *R. tropici* and related species and review genome-sequencing and gene-expression studies of two strains belonging to this group – *R. tropici* CIAT 899^T and of strain PRF 81, recently described as the type strain of the new species *R. freirei* – both of which have been successfully used in commercial inoculants for application to common-bean crops in the tropics.

74.2 PHYLOGENY AND TAXONOMY OF *R. tropici*

The report of *R. tropici* in 1991 (Martínez-Romero et al., 1991) represented the first description of a rhizobial species to include 16S-rRNA gene-sequence analysis. At that time, two types were recognized within the species, A and B. After two decades, accumulated data supported the description of the new species *R. leucaenae*, which included the strains

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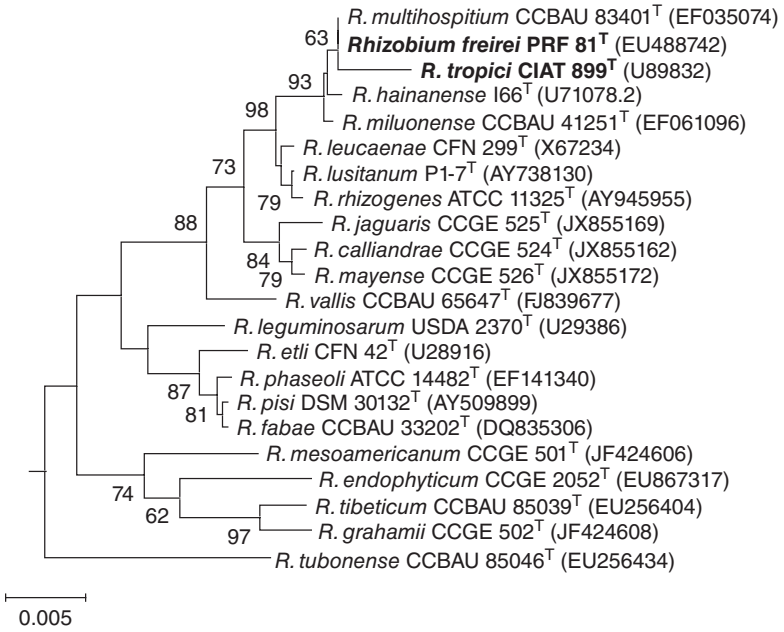

Figure 74.1 Neighbor-joining phylogeny of 16S rRNA gene sequences (1308 nt) of *Rhizobium tropici*, *R. freirei*, and other *Rhizobium* species. GenBank accession numbers are given within parentheses. Bootstrap support values based on 500 resamplings are shown at nodes only when they were ≥60%. Bar, 0.005 substitutions per nucleotide position.

previously classified as *R. tropici* type A (Ribeiro et al., 2012). In addition, evidence was generated of a new species within the strains classified as *R. tropici* (Ribeiro et al., 2009), and based on phenotypic and genomic data, the new species *R. freirei* was described for strain PRF 81^T. We must emphasize here that studies performed between 1991 and 2012 refer simply to *R. tropici*, whereas we now know that other related species are within this group; here we will refer exclusively to studies of symbionts of common bean and *Leucaena* species.

Other species are now recognized to be close relatives of *R. tropici*, including *Rhizobium lusitanum* (Valverde et al., 2006), *Rhizobium rhizogenes* (Hernández-Lucas et al., 2004; Velázquez et al., 2010), *Rhizobium multihospitium* (Han et al., 2008), and *Rhizobium miluonense* (Gu et al., 2008); the latter two, isolated in China, do not nodulate common bean. These rhizobia comprise closely related species, for which the designation "*R. tropici* group" has been proposed (Ribeiro et al., 2012).

Ribosomal sequences – with an emphasis on 16S rRNA – have become the basis of bacterial molecular phylogeny and taxonomy (Woese, 1987; Garrity and Holt, 2001). However, several species cannot be distinguished solely by analysis of their 16S rRNA, and strains within the "*R. tropici* group" fit clearly in this category (e.g., Han et al., 2008; Ribeiro et al., 2012). Strategies to improve species definition, such as the analyses of other ribosomal and housekeeping genes are discussed in more detail in Chapter 18. Here we will highlight that the use of the multilocus sequence analysis (MLSA) approach – considering other genes with evolution rates faster than that of 16S rRNA, but conserved enough to retain genetic information (e.g., Martens et al., 2007, 2008) – has been successfully used to define the taxonomy and phylogeny of strains within the "*R. tropici* group" (Ribeiro et al., 2009, 2012). Figure 74.1 shows the phylogeny based on MLSA of "*R. tropici* group" species and other rhizobial species, highlighting strains CIAT 899 and PRF 81.

Finally, we comment on the interesting resemblance of *R. tropici* with pathogenic agrobacteria (e.g., Lloret and Martinez-Romero, 2005), which has been confirmed in genomics (Ormeño-Orrillo et al., 2012) and proteomics (Gomes et al., 2012b) studies. This subject of study may help to elucidate evolutionary linkage between the processes of symbiosis and pathogenicity.

74.3 ORIGIN AND GEOGRAPHIC DISTRIBUTION OF *R. tropici*

R. tropici strain CIAT 899^T was isolated from a common-bean nodule in Colombia by an outstanding microbiologist, Dr. Peter H. Graham, when he was working at CIAT (Centro Internacional de Agricultura Tropical, International Center of Tropical Agriculture). However, the precise origin of *R. tropici* is not known, and a variety of ecosystems in Brazil have yielded the majority of the available strains, isolated both from common bean and *Leucaena* species (Martínez-Romero et al., 1991; Hungria et al., 1993, 1997, 2000, 2003; Mercante, 1993; Mercante et al., 1998; Straliotto et al., 1999; Andrade et al., 2002; Mostasso et al., 2002; Grange and Hungria, 2004; Pinto et al., 2007; Stocco et al., 2008).

It is worth mentioning that common bean is considered native to the Americas, and domestication is thought to

have taken place separately in two major centers of genetic diversification: the Mesoamerican center or northern group (from Mexico to the northern region of South America), and the Andean center or southern group (from southern Peru to the north of Argentina); a third minor domestication center may exist in Colombia (Gepts 1990; Kami et al., 1995), where CIAT 899^T was isolated. Wild common beans are not found in Brazil, but archeological evidences indicate that the legume was spread by migration and trade among Indian populations throughout history (Grange et al., 2007; Pinto et al., 2007). Therefore, the broad distribution of *R. tropici* in Brazil has at least two possible explanations: (i) as a symbiont of an indigenous legume that adapted to common bean when the legume was introduced and (ii) seeds traded from the Andean center of origin carried *R. tropici*, and its proliferation in Brazilian soils was favored by intrinsic properties of the species, such as tolerance of environmental stresses.

However, despite the strong evidence that *R. tropici* could have originated in Brazil, it is intriguing that the species has also been isolated from nodules of common bean and other legumes in Europe (e.g., in France, Geniaux et al., 1993; Amarger et al., 1994; and Spain, Herrera-Cervera et al., 1999), in Africa (e.g., in Kenya, Anyango et al., 1995; Odee et al., 2002; Tunisia, Mhamdi et al., 1999; Senegal and Gambia, Diouf et al., 2000), in Australia (e.g., Lafay and Burdon, 1998, 2001), in Central America (e.g., Mexico, Acosta-Durán and Martínez-Romero, 2002) and in North America (e.g., USA, Bernal et al., 2004). International trade in common-bean seeds carrying rhizobia (e.g., Andrade and Hungria, 2002) may have contributed to the distribution of the species, but it is noteworthy that *R. tropici* has also been reported as a symbiont of indigenous legumes far from South America (e.g., Australia, Lafay and Burdon, 1998, 2001).

In relation to other legume hosts, *R. tropici* strains have been isolated from *Gliricidia sepium* in Mexico (Acosta-Durán and Martínez-Romero, 2002) and Brazil (Menna et al., 2006; Binde et al., 2009), *Acaciella angustissima* in Mexico (Rincón-Rosales et al., 2009), *Mimosa scabrella* and *Mimosa caesalpiniifolia* in Brazil (Menna et al., 2006), *Bolusanthus* and *Aspartium* in Africa (Dagutat and Steyn, 1995), from native shrubby legumes in Australia (Lafay and Burdon, 1998) and *Lotus tenuis* in Argentina (Estrella et al., 2009). Furthermore, in studies of host range, *R. tropici* was found to nodulate *Macroptilium* sp. and several other legume hosts (Martínez-Romero et al., 1991; Hernández-Lucas et al., 1995; Hungria et al., 2000).

Altogether, these reports highlight *R. tropici* as being exceedingly promiscuous. One hypothesis to explain this broad host range is in terms of the largest number of Nod factors (or lipo-chitooligosaccharides, LCOs) described so far for a rhizobial species (Folch-Mallol et al., 1996).

74.4 INTERESTING FEATURES OF *R. tropici*

Although a variety of described and probably new rhizobial species have been isolated from common-bean nodules in Brazil, *R. tropici* (and related common bean and *Leucaena* species, *R. leucaenae* and *R. freirei*) is clearly dominant in field-grown plants, when both Andean and Mesoamerican genotypes are used as trap hosts (e.g., Hungria et al., 1997, 2000, 2003; Mostasso et al., 2002). This dominance may be attributed to intrinsic properties of the species, particularly tolerance of acidic and high temperature conditions (Martínez-Romero et al., 1991; Hungria et al., 1993, 2000, 2003; Graham et al., 1994). Strong competitiveness of *R. tropici* in acid soils has also been reported in Africa (Anyango et al., 1995).

Use of *R. tropici* related strains in inoculants for common-bean crops was suggested in light of its tolerance of environmental stresses, together with the important feature of higher stability of the pSym, in comparison to other common-bean rhizobia (Soberón-Chávez et al., 1986; Flores et al., 1988; Martínez-Romero et al., 1991; Segovia et al., 1993), probably due to the presence of a unique copy of the *nifH* gene (Martínez-Romero et al., 1991; Geniaux et al., 1993). It is essential to select genetically stable rhizobia for use in commercial inoculants, especially for countries, such as Brazil, where stressful environmental conditions frequently prevail. A selection program of *R. tropici* strains was started in Brazil in the mid-1990s, to identify strains with superior competitiveness and capacity to fix nitrogen. Since then, three strains have been recognized as very effective in fixing nitrogen with adequate industrial properties, and are now carried in thousands of doses of commercial inoculants: *R. tropici* CIAT 899^T (=SEMIA 4077), *Rhizobium freirei* PRF 81^T (=SEMIA 4080), and *R. tropici* H 12 (=SEMIA 4088); they are capable of supplying sufficient N to support grain yields of 2500 kg/ha or higher (Hungria et al., 2000, 2003). Under conditions not limiting to nitrogen fixation, for example, extremely acid soils (pH < 5.0) and severe drought, inoculated plants yield as much as plants receiving 60–100 kg of N-fertilizer/ha, and Figure 74.2 shows the results of one of these experiments. Interestingly, seed inoculation was successful even in the presence of a high indigenous population, contradicting one of the dogmas about failure of inoculation in soils with a high number of compatible rhizobia (e.g., Graham, 1981; Thies et al., 1991; Hardarson, 1993). Interestingly, in trials performed in Brazil, even in the presence of 10^3–10^6 indigenous rhizobia/g, soil nodule occupancy by elite inoculant strains was increased both by initial inoculation and by reinoculation, resulting in yield increases by 50% or higher (Hungria et al., 2000, 2003).

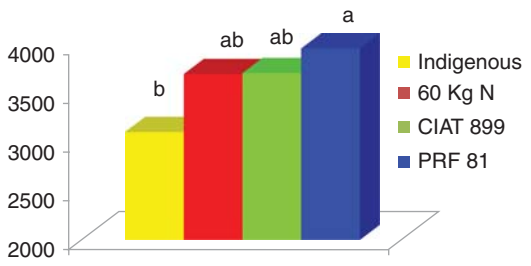

Figure 74.2 Grain yield (kg/ha) of common-bean cultivar Pérola non-inoculated, in soil with an indigenous population estimated at 10^5 cells/g soil; non-inoculated controls receiving or not N-fertilizer, split at sowing and flowering, or inoculated with *R. tropici* CIAT 899^T or *R. freirei* PRF 81^T at sowing. Experiment performed in Londrina, State of Paraná, Brazil.

74.5 GENOMICS

Recently, the genome of *R. tropici* CIAT 899^T was obtained and reported together with that of *R. freirei* PRF 81^T (Ormeño-Orrillo et al., 2012). Like other rhizobia, CIAT 899 and PRF 81 have multipartite genomes composed of several replicons. In both cases, one replicon has all of the ribosomal genes and all of the genes regarded as "essential," being thus a chromosome, whereas the remaining replicons have *repC*-type replication systems and are considered plasmids (three in CIAT 899 and four in PRF 81). These strains, and others ascribed to the "*R. tropici*" group (Acosta-Durán and Martínez-Romero, 2002; Pinto et al., 2007), possess fewer plasmids (two to four) in comparison to other rhizobia such as *R. leguminosarum*, which can have up to 11 (López-Guerrero et al., 2012).

Both strains CIAT 899 and PRF 81 possess a megaplasmid larger than 2 Mb, which can be classified as a chromid (Harrison et al., 2010) because its G + C content is similar to that of the chromosome and it harbors important genes that in other bacteria are located in the chromosome. Chromids seem to have important roles for survival in the soil and rhizosphere, as many rhizobial chromid genes are expressed in association with plants (Ramachandran et al., 2011; López-Guerrero et al., 2012). We have reported that chromids of CIAT 899 and PRF 81 contain several genes related to root and rhizosphere colonization and a large proportion of the transporter capacity of their genomes (Ormeño-Orrillo et al., 2012). Despite their importance, chromids can be cured under laboratory conditions, as has been reported for a megaplasmid-cured CIAT 899 derivative (Barreto and Baldani, 2002), probably because functions encoded in those replicons are dispensable in rich laboratory media.

CIAT 899 and PRF 81 share an almost identical pSym that has outstanding features such as possessing three *nodA* and five *nodD* genes, and also auxin- and gibberellin-biosynthesis genes (Ormeño-Orrillo et al., 2012). Reiteration of *nod* genes may explain the broad host ranges displayed by these strains. Genomic evidence indicates that this pSym is present also in *R. leucaenae* CFN 299^T (Ormeño-Orrillo et al., 2012) and in *R. tropici* WUR1 (Op den Camp et al., 2012). Other evidence, like plasmid profiles (Pinto et al., 2007) and *nod/nif* gene sequences – identical or almost identical to those of CIAT 899 – suggest that this pSym is present in several strains of the "*R. tropici*" group, including *Rhizobium* spp. (Zurdo-Piñeiro et al., 2004; Ibañez et al., 2008) and in species as *R. lusitanum* (Valverde et al., 2011) and other strains of *R. tropici* (Faghire et al., 2012). Most of these strains have been isolated from common bean and also from peanut (*Arachis hypogaea* L.) and wild legumes.

Plasmids other than the pSym have roles in nodulation and also encode important metabolic functions (for a recent review, see López-Guerrero et al., 2012). In CIAT 899, a derivative cured of its smallest plasmid (pRtrCIAT899a) induced fewer and smaller nodules in common bean than the wild type and was defective in nodulation competitiveness (Barreto et al., 2012). The defective phenotype of this cured derivative may be explained by the loss of pRtrCIAT899a *teu* genes that are important for common-bean nodulation (Rosenblueth et al., 1998). PRF 81 also has *teu* genes in one of its smaller plasmids (pPRF81b). Other genes of pRtrCIAT899a that may influence symbiosis include homologs of *yagTSR* involved in purine metabolism and a tripartite ATP-independent periplasmic (TRAP)-family dicarboxylate transporter. The two smallest plasmids of PRF 81 also carry genes that may be related to symbiosis, including an aspartate ammonia-lyase, sarcosine catabolism genes, an alpha-glucoside utilization locus in pRF81a, two TRAP transporters, a polyhydroxybutyrate metabolism gene, and an aspartate aminotransferase in pPRF81b.

The genomic analyses also emphasized the potential for stress tolerance in CIAT 899 and PRF 81. Analyses of the predicted gene functions in the genomes of both strain revealed the potential for resistance to or tolerance of unfavorable conditions such as high temperatures, low pH, high heavy-metal concentrations, as well as stressful osmotic, oxidative, and nitrosative conditions (Ormeño-Orrillo et al., 2012). As seen in Table 74.1, most of the genes that can be related to stress tolerance are located in the chromosomes of both strains; nevertheless, a large proportion of the genes is located in their megaplasmid chromids. More than 33% of all genes related to osmotolerance and oxidative stress response, and all involved in nitrosative stress, are located in the chromids. The proportion is even higher for genes related to heavy-metal tolerance, 44% and 67% in CIAT 899 and PRF 81, respectively. Chromids, having intermediate features between chromosomes and plasmids, are considered stable replicons (Harrison et al., 2010) and may be less subject to horizontal gene transfer. Finding the majority of genes related to stress response in the chromosome/chromid seems to indicate that resistance to abiotic stresses is an intrinsic

Table 74.1 Number of genes involved in stress response in each replicon of *R. tropici* CIAT 899^T and *R. freirei* PRF 81^T

	Number of Genes in Each Replicon						
	CIAT899				PRF81*		
Stress	Chr	pC	pB†	pA	Chr	pD	pC†
High temperature	14	2	2	–‡	14	3	2
pH	21	8	–	2	21	7	–
Osmolarity	21	11	1	–	19	12	1
Oxidative	13	9	1	–	13	8	1
Nitrosative	–	1	–	–	–	3	–
High metal concentration	9	7	–	–	5	10	–

*No genes were identified in PRF 81 plasmids pB and pC.

†Symbiotic plasmid.

‡"–" meaning no genes.

characteristic of these strains that may not be lost when a plasmid is cured. The pSym also carries stress-related genes (Table 74.1), an observation that is consistent with evidence that rhizobia are stressed at some stage(s) of formation of, and/or functioning in, root nodules.

74.6 PROTEOMICS

The term "proteome" refers to the set of proteins expressed by a cell, tissue, or organism in a specific condition; the importance of these studies relies also in their dynamics, as changes may occur with the physiologic state of cells, or in response to internal and external stimuli. Accordingly, proteomic tools were developed to study a large number of proteins, allowing a global view of protein expression (Graves and Haystead, 2002).

Most proteomics studies performed with rhizobia have employed 2D electrophoresis to separate proteins. By this approach, proteins are submitted, in the first step, to isoelectric focusing (IEF), followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), generating bidimensional maps for the samples, where proteins are separated by their isoelectric points (pIs) and molecular weights (MWs) (Westermeier and Naven, 2004). In addition, other important information can be obtained from the gels, including expression rates and relative amounts of proteins (Cash, 1998; Pandey and Mann, 2000; Herbert et al., 2001; Jungblut, 2001).

A proteomics reference map of *R. freirei* PRF 81 grown *in vitro* revealed 150 spots, from which 115 were identified, representing 109 different proteins (Gomes et al., 2012b). Interestingly, the great majority of the proteins identified showed highest genetic similarity with *Agrobacterium* (=*Rhizobium*) species, in particular *R. radiobacter*, confirming the genomic data obtained with this strain (Ormeño-Orrillo et al., 2012).

In the reference map of PRF 81, chaperonin GroEL, elongation factor (EF) Tu, dipeptide ATP-binding cassette (ABC) transporter, and beta subunit of F0F1 adenosine triphosphate (ATP) synthase were the protein spots with highest total volumes (Gomes et al., 2012b). In the functional classification of the proteins according to clusters of orthologous groups (COGs), proteins were distributed into 17 categories (Fig. 74.3), belonging to four functional groups: metabolic function, cellular processes and signaling, information storage and processing, and poorly characterized proteins. Of the identified proteins, 51% were related with metabolic function, a key feature that could strongly contribute to the high capacity of the strain to survive in limiting environmental conditions common in the tropics.

Proteins that participate directly or indirectly in the symbiosis were also detected in the proteomic reference map of PRF 81 (Gomes et al., 2012b). Among them, glutamine synthetase I (GS I), with a role in biological nitrogen fixation confirmed in studies with auxotrophic mutants, where the mutant GS I was defective in the ability to derepress nitrogenase, being inefficient in fixing nitrogen (Kondorosi et al., 1977; Ludwig and Signer 1977).

Among the transcription factors of PRF 81, emphasis was given to the expression of LysR family transcriptional regulatory proteins (Gomes et al., 2012b). The LysR transcription regulators have a key role in signal exchanges required for plant root-hair infection, probably through the regulation of *nod*-gene expression (Doty et al., 1993; Luo et al., 2005). Several other proteins related to the exchange of molecular signals between the host legume and the symbiont were also identified in PRF 81, including the two-component regulatory system ChvI, which positively regulates the transcription of *exo* genes that encode enzymes for succinoglycan exopolysaccharide (EPS) biosynthesis in response to plant chemical signals (Cheng and Walker, 1998; Chang et al., 2002). In addition, this EPS may also

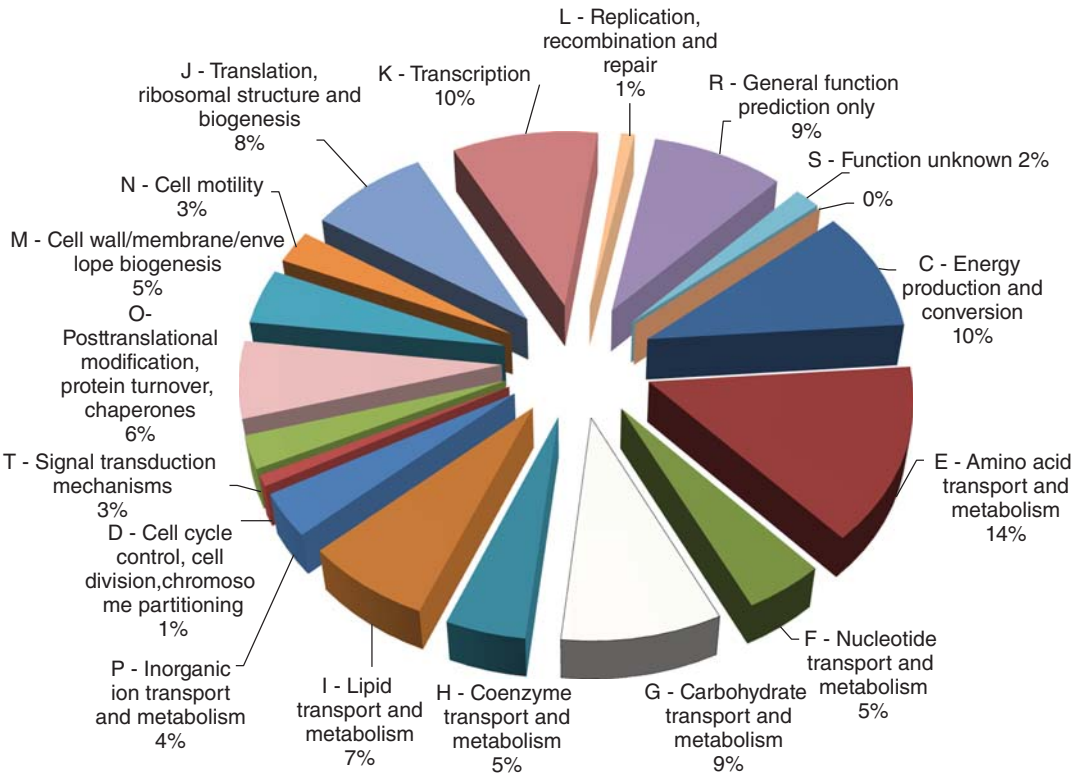

Figure 74.3 COG classes of proteins identified in the reference map of *R. freirei* PRF 81^T. From Gomes et al. (2012b).

be related to the suppression of the plant-defense response during the process of root infection (Cheng and Walker, 1998; Chen et al., 2009).

Beyond reference maps, proteomic analysis is applicable to generate a global view of responses to specific conditions; this approach, differential proteomic analysis, has been employed to elucidate peculiarities of organisms. The higher tolerance of strain PRF 81 to heat stress was investigated using this approach, and 59 proteins were found to be upregulated when exposed to high temperature (37 °C) (Gomes et al., 2012a). Heat-induced proteins identified in PRF 81 included examples that are highly conserved among bacterial species, designed for rapid adaptation to environmental and metabolic changes, including molecular chaperones such as DnaK and GroEL (Gomes et al., 2012a), which are polypeptide-binding proteins implicated in protein folding, protein targeting to membranes, renaturation, and in the control of protein–protein interactions (Wagner et al., 2009).

Still with reference to PRF 81, it is worth mentioning the upregulation of translation factors, with three EFs (EF-Tu, Ef-G, and Ef-Ts) and one initiation factor (IF-2) (Gomes et al., 2012a). In addition to their main function of ensuring gene expression accuracy by transporting the correct codons in the translation process, several authors have reported that elongation and IFs can also act as chaperones in response to heat stress (Fayet et al., 1989; Caldas et al., 1998), suggesting that they might be ancestral protein-folding factors against heat-stress damage (Caldas et al., 2000).

Interaction among groups of proteins with different functions is another important aspect that differential proteomics studies can highlight. One of the main findings from PRF 81 heat-stress proteomic data was the cross talk between heat and oxidative stresses, and several antioxidant factors were identified when PRF 81 was grown at high temperatures (Gomes et al., 2012a). Oxidative stress is characterized by high levels of reactive oxygen species (ROS) that are by-products of normal metabolic processes and may be lethal for cells. Previous studies (Kopcinska, 2009; Meilhoc et al., 2011; Peleg-Grossman et al., 2012) reported the accumulation of ROS in early stages of establishment of the *Rhizobium*/legume symbiosis, acting as specific signals (Nanda et al., 2010); however, they can also be toxic to the bacterium. To detoxify ROS, symbiotic bacteria display multiple defense mechanisms that are required for both the development and the functioning of the symbiosis (Pauly et al., 2006). Reactivation of proteins damaged by oxidative stress is one of the strategies to minimize ROS effects, a function displayed by thioredoxin (TrxA). Additionally, Fernando et al. (1992) reported an indirect action against oxidative stress performed by bacterioferritin (Bfr), which transports inorganic ions such as Fe^{2+}, resulting in the

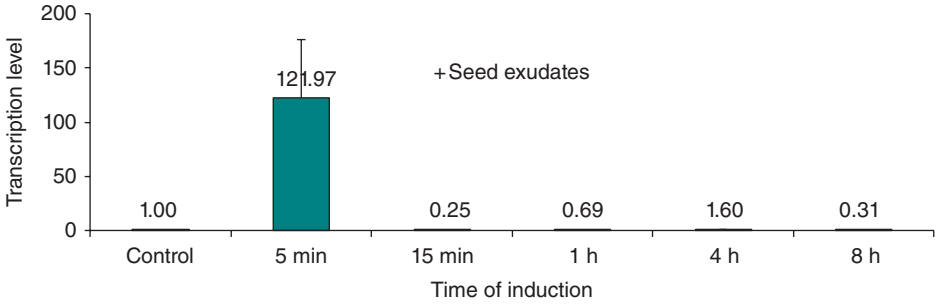

Figure 74.4 Transcription of *nodC* gene of *R. freirei* PRF 81^T grown until the exponential phase and then incubated with seed exudates for various periods of time. Data represent the means of three biological replicates, each with three replicates ± SD. Relative quantification (RQ) was estimated by the equation $1.95^{\Delta\Delta Ct}$. From Oliveira et al. (2010).

decomposition of peroxides overproduced during the oxidative stress. Other proteins, such as isocitrate dehydrogenase, play a key role in NADPH recycling under oxidative stress (Patridge and Ferry, 2006; Marino et al., 2007; Brown et al., 2010). All these antioxidant proteins were upregulated in PRF 81 at high temperatures (Gomes et al., 2012a), highlighting a finely tuned mechanism conferring stress tolerance.

Differential proteomic analysis was also applied in a study with *R. tropici* CIAT 899 to investigate the effects of acidity (Peick et al., 1999). A pH shift from 6.8 to 5.2 affected the expression of five pH-induced proteins, PipA, B, C, D, and E, and of four pH-reduced proteins, PrpA–D. PipA and B proteins were then selected for N-terminal sequencing; PipA showed high similarity with several bacterial enzymes involved in modifications in cells, mainly epimerization of glucosenucleosides (Peick et al., 1999). The highest similarity (68%) was found with PipA of *Staphylococcus aureus*, which is involved in the biosynthesis of *N*-acetylgalactosamino uronic acid, a component of staphylococcal type I capsules (Lin et al., 1994). PipA also showed similarity (66%) with a uridine diphosphate (UDP)-glucose epimerase from *Sinorhizobium meliloti*, a protein involved in EPS biosynthesis and important to biofilm formation and cell protection (Yao et al., 2004; Fujishige et al., 2006).

We conclude that, despite the few proteomic studies performed so far with *R. tropici*-group strains, important results have been obtained with both the proteomic reference map and differential proteomic experiments, helping to elucidate mechanisms related to the stress tolerance of the species.

74.7 TRANSCRIPTOMICS

Novelties have also been revealed in transcription studies. In *R. freirei* PRF 81, three genes related to nodulation and located in the pSym were investigated by reverse-transcription quantitative polymerase chain reaction (RT-qPCR): *nodC* (with a role in the biosynthesis of the basic structure of the chito-oligosaccharides, also an important determinant of LCO chain length), *nodG* (encodes a 3-oxoacyl-[acyl] carrier protein reductase involved in the biosynthesis and transfer of common fatty acids), and *glgX* (involved in the transformation of glycogen into 1,4-glycan, and located immediately upstream of *nodN* in *R. tropici*) (Oliveira et al., 2010). All three genes showed low levels of transcription when the cells were grown into the exponential phase in the presence of common-bean-seed exudates or of the root *nod*-gene inducer naringenin. However, when cells in the exponential phase of growth were incubated with seed exudates, transcription occurred after only 5 min, and *nodC*, *nodG*, and *glgX* were transcribed 122-, 15-, and 50-fold more than the control, respectively (Fig. 74.4), followed by a rapid overall decrease in gene transcription. Much lower levels of transcription were observed in the presence of naringenin, and maximum transcription in the presence of this flavonoid required 8 h of incubation for all three genes (Oliveira et al., 2010). The results could be interpreted as leading to an initial high production of Nod-factor synthesis enzymes, which, very rapidly, is reduced to much lower levels, and another hypothesis is that this mechanism prevents inhibition of nodulation by an excessively high concentration of Nod factors. The results also indicate that compounds in seed exudates – still to be determined – may be responsible for rapid induction of *nod* genes. The extremely rapid transcription described in this study (e.g., Fig. 74.4) indicates that signal exchanges between host legumes and rhizobia may occur considerably more quickly than previously thought (Oliveira et al., 2010).

Another transcription study was performed with three biosynthetic polysaccharide genes of PRF 81, *lpxA*, *lpxE* (involved in the biosynthesis and modification of the lipid-A anchor of lipopolysaccharide), and *rkpI* (involved in the synthesis of a lipid carrier required for the production of capsular polysaccharides). RT-qPCR analysis revealed, for the first time, that inducers released from common-bean seeds strongly stimulated the expression of all three genes (Oliveira et al., 2013). Again, the highest increases in the transcription rates – of about 50-fold for *lpxE* and about 30-fold for *lpxA* and *rkpI* – were observed after only 5 min of incubation. Upregulation of *lpxE*, *lpxA*, and *rkpI* genes suggests that inducer compounds in seed exudates can modulate the biosynthesis and modification of the surface

polysaccharides of PRF 81, leading to cell-wall changes that are implicated in the establishment of the symbiosis (Oliveira et al., 2013).

74.8 CONCLUDING REMARKS

In this chapter, we pooled information about biodiversity of bacteria related to the *R. tropici* species (including the recently described *R. leucaenae* and *R. freirei*). Among the main features of *R. tropici* and related species are a broad host range and high tolerance to environmental stresses. Two elite strains showing high capacity of fixing nitrogen and high competitiveness in the tropics were included in our studies, *R. tropici* CIAT 899^T and *R. freirei* PRF 81^T, both commonly applied in commercial inoculants to seeds of common bean prior to planting. High grain yields can be obtained by inoculation and reinoculation with those strains, even in soils with abundant indigenous compatible rhizobia. Interesting information about these two strains has been revealed by genome sequencing, with an emphasis on a highly conserved pSym, which may help to explain similarities in the stability of symbiotic properties. Proteomic studies have highlighted new proteins, brought interesting evolutionary information, and identified proteins related to heat and acid stresses. Transcription studies with genes related to nodulation and surface polysaccharide biosynthesis in PRF 81 indicated a remarkably rapid transcription in the presence of common-bean-seed exudates, with no parallel with any other report with rhizobia. In summary, exciting information obtained in the few studies performed so far emphasize how useful these bacteria are.

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Chapter 75

The *Frankia alni* Symbiotic Transcriptome

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75.1 INTRODUCTION

Frankia alni and its host, *Alnus glutinosa* establish a nitrogen-fixing root symbiosis based on an exchange of signals and metabolites (see Chapters 35, 42, 43, 48, 55). Knowledge of the determinants involved has however been hampered by the lack of a genetic transformation system despite numerous attempts with protoplasts regeneration (Normand et al., 1987; Tisa and Ensign, 1987) and electroporation (Cournoyer and Normand, 1992; Myers and Tisa, 2003). The reasons for this failure are unknown but may have to do with the slow growth rate of *Frankia*, the presence of a restriction and modification system, thick wall with unusual lipids or with specific promoters. A complementation of *nodBC Rhizobium* mutants with *Frankia alni* DNA also failed (Ceremonie et al., 1998).

On the plant side, recent progress has been made. It has been shown that a kinase, SymRK homologous to the one that plays a crucial role in legumes by detecting Nod factors, is also necessary for nodulation of *Casuarina* (Gherbi et al., 2008) and *Datisca* (Markmann et al., 2008; see Chapter 55). Furthermore, an expressed sequence tag (EST) study of *Casuarina* and *Alnus* showed most of the determinants known in legumes were present in actinorhizal plants (Hocher et al., 2011). Actinorhizal host plants thus appear to have a symbiotic cascade very similar to the one existing in legumes (see Chapter 55).

The genomes of several symbiotic *Frankia* strains (see Chapter 24) were sequenced among other things to identify *nod* genes (Normand et al., 2007b); however, no canonical *nod* clusters (common *nod*ABC) could be identified (Normand et al., 2007a). It was thus decided to use a transcriptomic approach to try to identify groups of genes involved in symbiosis (Alloisio et al., 2010).

75.2 MATERIALS AND METHODS

Frankia alni ACN14a was grown at 28 °C with stirring at 200 rpm in defined BAP (Basal medium with propionate) containing 5 mM ammonium chloride (N-replete condition) and 5 mM sodium propionate as carbon source. Seeds of *Alnus glutinosa* were planted and grown for 5–10 weeks under fluorescent lighting with a 16-h light and 8-h nycthemeral regime at 21/25 °C. Seedlings were transferred to plastic pots (8 seedlings/pot) and grown for 4–9 weeks with 0.5 g of KNO_3 per liter and, then, for 1 week without KNO_3. *F. alni* ACN14a cells derived from 75 ml of log-phase culture were inoculated to a pot. Root nodules (≤3 lobes) were harvested

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into liquid nitrogen 3–4 weeks after inoculation and stored at −80 °C. Nodules from about 24 plants per replicate were pooled for RNA extraction, and three biological replicates were treated (Alloisio et al., 2010).

RNA was purified from bacterial cells and nodules using RNeasy plant mini kit (Qiagen) and on-column DNA digestion with the RNase-free DNase set (Qiagen). In order to remove any contaminating DNA, a second DNase treatment was performed with RQ1 RNase-free DNase (Promega, Charbonnières-les-Bains, France), followed by RNA cleanup using RNeasy mini-kit. Purity, concentration, and quality of RNA samples were checked using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Courtaboeuf, France) and agarose gel electrophoresis. Based on the sequence and annotation data for *F. alni* ACN14a, a whole-genome high-density microarray was designed and manufactured by Roche NimbleGen, Inc. (Madison, WI, USA). Due to high guanine-cytosine (GC) content (72.8%) of the *F. alni* genome, a 60-mer oligo length was chosen for probe design. NimbleGen design rules and analysis algorithms found probes for 6,607 genes out of 6786. There were 18 probes per gene obtained for 6340 genes and <18 probes per gene were obtained for 267 genes. Probes were duplicated on the array (Alloisio et al., 2010).

75.3 RESULTS

Overall, transcript levels were compared between young nodules (3–4 weeks old) and cultured cells grown in N-replete minimal medium, and it permitted to show that in one data set (A), 871 genes showed a differential expression in symbiosis versus *in vitro* N-replete free-living (FL) cells compared with 457 genes in the other data set (B). In the two data sets, there were 292 "common" genes, with 166 genes upregulated and 126 genes downregulated (Table 75.1). There were 183 of these genes that belonged to the core genome defined with the three first published genomes (ACN14a, CcI3, and EaN1pec). The highest upregulation was for the *nifHDK* genes that were 210–270 times upregulated in symbiosis.

75.3.1 Genes Known to be Involved in Symbiosis

There are several genes known to be involved in symbiosis comprising the *nif*, *hup*, *suf*, and *isp* + *shc* genes. The whole *nif* cluster that comprises 18 genes from FRAAL6797 (ferredoxin) to FRAAL6814 (*nifV*) was the most upregulated, while neighboring genes of unknown function were not. In the cluster, the structural genes for the nitrogenase, *nifD*, *K*, and *H*, were more upregulated than the ancillary nitrogenase genes. Another ferredoxin FRAAL3855, situated away from the *nif* cluster and postulated to provide the nitrogenase enzyme with reductants, was also highly upregulated.

Hydrogenases permit to recycle hydrogen, a byproduct of nitrogenase. Hydrogenase cluster #2 (hup2) that comprises 11 genes from FRAAL1822 (protein of unknown function or puf) to FRAAL1832 (puf) was for the most part upregulated but only at a fraction of the *nif* genes. The most upregulated *hup* gene FRAAL1830, hupS2 was 12.45-fold upregulated, less than one-tenth of the *nif* structural genes. The second hydrogenase cluster, hup1 comprising FRAAL2388–2395 was not upregulated in symbiosis.

The *suf* cluster that provides iron–sulfur cluster likely for nitrogenase and hydrogenases ranges from FRAAL4557 to FRAAl4563. The *suf* genes are upregulated at levels comparable to those of the *hup*2 genes. The most upregulated was *sufC*, upregulated 7.45-fold.

The shc1 cluster (FRAAL1428–1435) contains the major genes leading to hopanoid biosynthesis such as the *shc* gene encoding the squalene hopene cyclase and yielding the polycyclic lipids that in vesicles protect nitrogenase from oxygen. In this cluster, two genes were significantly upregulated and a squalene/phytoene dehydrogenase (FRAAL1430, fold change (fc) = 5.25). The genes in the initial part of the non-mevalonate pathway are clustered away from *shc*1 but most are nevertheless upregulated, such as *ispG* (FRAAL5772, fc = 6.65), *dxs* (FRAAL2088, fc = 5.9), or *idi* (FRAAL6504, fc = 4.20). A second *shc* gene, *shc*2, situated away from all these genes (FRAAL2491) was not upregulated.

75.3.2 Homologs of *nod* Genes

There are no significant homologs of *nodA* in *Frankia alni*, however, there are three homologs of *nodB* (FRAAL4911, FRAAL4175, FRAAL1746) and six homologs of *nodC* (FRAAL6263, FRAAL6103, FRAAL2582, FRAAL0797, FRAAL1439, FRAAL2169), the gene that codes for a glycosyl transferase that oligomerizes *N*-acetyl-glucosamine into the backbone of the Nod factor (Normand et al., 2007a) and none of these distant homologs was upregulated above the significance threshold.

75.3.3 Secondary Metabolites

Frankia genomes have been shown to contain several secondary metabolites determinants (Udwary et al., 2011). These genes are for the most part not modified upon switch to symbiosis, except for the hopanoid cluster that is upregulated, a spore pigment cluster, a Type I iterative PKS (polyketide synthase). The other genes were unmodified (Table 75.2).

75.3.4 Transporters

Exchange of nutrients between the microbe and the host plant implies in particular photosynthates that the plant feeds the microbe with and fixed nitrogen that the microbe yields

Table 75.1 The 292 genes differentially expressed in nodules versus free-living cells

Gene Number*	Gene Name*	Product Description*	Fold-Change†	COG ID‡	Core Genes§	Specific Genes**	Synteny††	Core Secretome‡‡
FRAAL6812	*nifD*	Nitrogenase alpha-subunit	269.80	C	+	+	+	–
FRAAL6813	*nifH*	Nitrogenase reductase	250.05	DP	+	+	+	–
FRAAL6811	*nifK*	Nitrogenase beta-subunit	209.65	C	+	+	+	–
FRAAL6814	*nifV*	Homocitrate synthase	113.15	CE	+	+	–	–
FRAAL3855	*fdxI*	Ferredoxin	98.50	C	+	–	–	–
FRAAL6806		cpuf	77.20		+	+	+	–
FRAAL6807		cpuf	65.50		+	+	+	–
FRAAL6805	*nifW*	Nitrogenase stabilizing/protective protein	56.30		+	+	+	–
FRAAL6808	*nifX*	NifX protein	56.25		+	+	+	–
FRAAL6810	*nifE*	Nitrogenase iron-molybdenum cofactor biosynthesis protein	48.05	C	+	+	+	–
FRAAL6797		Ferredoxin	44.30	C	+	–	+	–
FRAAL6799		Ferredoxin oxidoreductase alpha-subunit	33.90	C	+	–	+	–
FRAAL6803	*nifB*	FeMo cofactor biosynthesis protein	28.80	R	+	+	+	–
FRAAL6801		Putative molybdopterin biosynthesis protein HesA	27.00	H	+	+	+	+
FRAAL3254		puf	26.60	S	+	+	–	–
FRAAL6816		puf	25.85	E	+	+	+	–
FRAAL6486	*groL*	Chaperone Hsp60	23.75	O	+	–	–	–
FRAAL6804	*nifZ*	NifZ protein	22.20	C	+	+	+	–
FRAAL3287		cpuf	16.70		–	–	–	–
FRAAL1426		puf	16.45		–	–	–	–
FRAAL6798		Ferredoxin oxidoreductase beta-subunit	16.40	C	+	–	+	–
FRAAL4471		puf	14.30	NT	–	–	–	–
FRAAL6541		puf	14.05		–	–	–	–
FRAAL4403		Putative DEAD-box RNA helicase	13.10	LRJK	+	–	+	–
FRAAL4419		puf	13.00		–	–	–	–
FRAAL3922		Putative export protein	12.90	RU	–	–	–	–
FRAAL4897		1-Acylglycerol-3-phosphate *O*-acyltransferase	12.65	I	+	–	–	–
FRAAL2922		puf	12.50		–	–	–	–
FRAAL1830	*hupS2*	[NiFe] uptake hydrogenase small subunit	12.45	C	–	–	–	–
FRAAL4473		puf	12.35		–	–	–	–
FRAAL5232		Putative integration host factor-like protein	12.15	L	+	–	+	–
FRAAL1156	*sucC*	Succinyl-CoA synthetase beta-subunit	12.05	CI	+	–	+	–
FRAAL4896		Putative multidrug efflux transporter	11.55	RUV	+	–	+	–
FRAAL2921		puf; putative repressor-like DNA-binding domain	11.35		–	–	+	–
FRAAL4858		Putative LuxR-family transcriptional regulator	11.00	K	+	–	–	–
FRAAL1673		Putative molybdenum-binding protein	10.80	GR	+	–	–	–
FRAAL5724		Putative RNA polymerase ECF sigma factor	10.65	K	+	–	+	–
FRAAL6657		*N*-Acetylmuramoyl-L-alanine amidase (autolysin)	10.50	M	+	–	+	+
FRAAL1829	*hupL2*	[NiFe] uptake hydrogenase large subunit	10.40	C	–	–	+	–

(*continued*)

Table 75.1 (*Continued*)

Gene Number*	Gene Name*	Product Description*	Fold-Change†	COG ID‡	Core Genes§	Specific Genes**	Synteny††	Core Secretome‡‡
FRAAL4147	*ctaD*	Cytochrome *c* oxidase	10.25	C	+	−	−	−
FRAAL0111	*gltA*	Citrate synthase	9.75	C	+	−	+	−
FRAAL1831	*hypA2*	Hydrogenase nickel incorporation protein	8.95	R	−	−	+	−
FRAAL5516		Putative AAA family cell division control ATPase	8.80	OKR	−	−	−	−
FRAAL1189	*sdhD*	Succinate dehydrogenase hydrophobic membrane anchor protein	8.70	C	−	−	−	−
FRAAL1157	*sucD*	Succinyl-CoA synthetase alpha-subunit	8.60	C	+	−	+	−
FRAAL0774		puf	8.05		−	−	−	−
FRAAL1427		Putative short-chain dehydrogenase/oxidoreductase	8.00	IQR	+	−	+	+
FRAAL5604		puf	7.90		−	−	−	−
FRAAL1155		Putative serine/threonine protein kinase	7.85	KLRT	+	+	+	−
FRAAL1832	*hypB2*	Hydrogenase nickel incorporation protein	7.60	EKO	+	−	+	−
FRAAL5231	*gmk*	Guanylate kinase	7.60	F	+	−	+	−
FRAAL4560	*sufC*	Transport protein associated with Fe–S cluster assembly	7.45	EPV	+	−	+	−
FRAAL2920		Putative GNAT-family acetyltransferase	7.40	KRJ	+	+	+	−
FRAAL4559	*sufS*	Cysteine desulfurase/selenocysteine lyase	7.40	ER	+	−	+	−
FRAAL2240		puf	7.35		−	−	−	−
FRAAL6153	*sthA*	Soluble pyridine nucleotide transhydrogenase	7.30	RE	+	+	+	−
FRAAL1544		Putative LuxR-family transcriptional regulator	7.10	K	+	+	+	−
FRAAL5046		puf	7.10		−	−	−	−
FRAAL5725		puf	7.10		−	−	−	−
FRAAL4195		Conserved hypothetical transmembrane protein	7.05	FE	−	−	−	−
FRAAL1435		cpuf	6.90	RH	+	−	+	−
FRAAL2064	*acnA*	Aconitate hydratase 1	6.85	EC	+	−	+	−
FRAAL4561	*sufE*	Ferredoxin-containing [2Fe-2S] Rieske domain	6.70	PR	+	−	+	−
FRAAL5772	*ispG*	1-Hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase	6.65	I	+	−	+	−
FRAAL5933	*atpA*	Membrane-bound ATP synthase, F1 sector, alpha-subunit	6.55	C	+	−	+	−
FRAAL6604		Putative RNA polymerase ECF-subfamily sigma factor	6.45	K	−	−	−	−
FRAAL1190	*sdhC*	Succinate dehydrogenase membrane subunit	6.30	C	−	−	−	−
FRAAL5122	*cyoB*	Cytochrome *c* oxidase subunit I	6.30	C	+	−	+	−
FRAAL5603		Putative integral membrane protein	6.30		+	−	+	−
FRAAL1097	*rplR*	50S ribosomal subunit protein L18	6.20	J	+	−	+	+
FRAAL6192		Putative adenylate kinase	6.05	F	+	+	+	−
FRAAL1988		Putative secreted protein	6.05		−	−	−	−
FRAAL1090	*rpsQ*	30S ribosomal protein S17	6.00	J	+	−	+	−
FRAAL6538		puf	6.00	JIQ	−	−	−	−

Table 75.1 (*Continued*)

Gene Number*	Gene Name*	Product Description*	Fold-Change†	COG ID‡	Core Genes§	Specific Genes**	Synteny††	Core Secretome‡‡
FRAAL0711		Putative MarR-family transcriptional regulator	5.95	K	+	–	+	–
FRAAL3350	*cysK*	Subunit of cysteine synthase A and *O*-acetylserine sulfhydrolase A	5.95	E	+	–	–	–
FRAAL2088	*dxs*	1-Deoxy-D-xylulose-5-phosphate synthase 2	5.90	CHI	+	–	+	–
FRAAL5931	*atpD*	Membrane-bound ATP synthase, F1 sector, beta-subunit	5.90	C	+	–	+	–
FRAAL1091	*rplN*	50S ribosomal subunit protein L14	5.85	J	+	–	+	–
FRAAL6594		puf	5.65		–	–	–	–
FRAAL1089	*rpmC*	50S ribosomal protein L29	5.45	J	+	–	+	–
FRAAL2298	*folD*	Methylenetetrahydrofolate dehydrogenase/cyclohydrolase	5.40	H	+	–	+	–
FRAAL5620		cpuf	5.40	K	+	–	+	–
FRAAL5936	*atpE*	ATP synthase C chain	5.40	C	+	–	+	–
FRAAL0694		puf	5.35		–	–	–	–
FRAAL1682		puf	5.35		–	–	–	–
FRAAL1093	*rplE*	50S ribosomal subunit protein L5	5.25	J	+	–	+	–
FRAAL1430		Putative squalene/phytoene dehydrogenase	5.25	HRJ	+	–	+	–
FRAAL1094	*rpsN*	30S ribosomal protein S14-1	5.20	J	+	–	+	–
FRAAL5932	*atpG*	Membrane-bound ATP synthase, F1 sector, gamma-subunit	5.15	C	+	–	+	–
FRAAL5287		Putative glycosyl transferase group 1	5.10	M	+	+	+	–
FRAAL3757		puf	5.05	S	+	+	+	–
FRAAL1078	*fusA*	Protein-chain elongation factor EF-G	4.95	J	+	–	+	–
FRAAL5121		Putative integral membrane protein	4.95	EGPR	+	–	+	–
FRAAL1835		puf	4.95		–	–	–	–
FRAAL1491		Putative HTH-type transcriptional regulator	4.85	K	+	+	+	–
FRAAL5123	*cyoA*	Cytochrome *c* oxidase subunit II	4.80	C	+	–	+	–
FRAAL4553	*glgC*	Glucose-1-phosphate adenylyltransferase	4.80	JM	+	–	+	+
FRAAL1539		puf	4.60		–	–	–	–
FRAAL6693	*gdhA*	Glutamate dehydrogenase (GDH)	4.55	JE	+	+	+	–
FRAAL5935	*atpF*	ATP synthase B chain	4.50	C	+	–	+	–
FRAAL6584	*ppa*	Inorganic pyrophosphatase	4.45	C	+	–	+	–
FRAAL1098	*rpsE*	30S ribosomal protein S5	4.30	J	+	–	+	–
FRAAL1099	*rpmD*	50S ribosomal subunit protein L30	4.30	J	+	–	+	–
FRAAL1109	*rpsD*	30S ribosomal subunit protein S4	4.25	J	+	–	+	–
FRAAL5985		Malate dehydrogenase	4.25	C	+	–	+	–
FRAAL5992	*sigH*	SigH putative RNA polymerase sigma factor	4.25	K	+	–	+	–
FRAAL1050		Putative oxidoreductase beta-subunit	4.20	CH	+	–	+	–
FRAAL1092	*rplX*	50S ribosomal subunit protein L24	4.20	J	+	–	+	–
FRAAL5930	*atpC*	ATP synthase epsilon chain	4.20	C	+	–	+	–
FRAAL6817		puf	4.20		–	–	–	–
FRAAL6504	*idi*	Isopentenyl diphosphate isomerase	4.20	ILR	+	+	–	–
FRAAL5779	*tsf*	Elongation factor Ts	4.15	J	+	–	+	–
FRAAL6085		Mrp protein homolog	4.15	DR	+	–	+	–
FRAAL0599	*cydB*	Cytochrome *bd* ubiquinol oxidase subunit II	4.10	C	+	+	+	–
FRAAL4934	*fadA*	3-Ketoacyl-CoA thiolase	4.10	IQ	+	–	+	–

(*continued*)

Table 75.1 (*Continued*)

Gene Number*	Gene Name*	Product Description*	Fold-Change†	COG ID‡	Core Genes§	Specific Genes**	Synteny††	Core Secretome‡‡
FRAAL5654	*sdhA*	Succinate dehydrogenase flavoprotein subunit A	4.10	CER	+	−	+	−
FRAAL5612	*mdh*	Malate dehydrogenase	4.10	C	−	−	−	−
FRAAL5799		puf	4.00		+	+	+	−
FRAAL1051		Putative oxidoreductase alpha-subunit	3.90	C	+	−	+	−
FRAAL5991	*rshA*	Anti-sigma factor	3.90	T	+	−	+	−
FRAAL6152	*fumC*	Fumarase C	3.90	EC	+	−	+	−
FRAAL5152	*sucB*	Dihydrolipoamide succinyltransferase	3.85	CI	+	−	+	−
FRAAL5759		cpuf	3.85	S	+	−	+	−
FRAAL5807		cpuf	3.80	R	+	−	+	−
FRAAL5803		puf	3.80		−	−	−	−
FRAAL5773		Hypothetical zinc metalloprotease	3.75	MR	+	−	+	−
FRAAL6429		puf	3.75	M	+	+	+	−
FRAAL5791	*rimM*	16S rRNA processing protein	3.70	J	+	−	+	−
FRAAL1188	*sdhA*	Succinate dehydrogenase flavoprotein subunit	3.70	C	−	−	−	−
FRAAL5517		cpuf	3.70	H	+	−	−	−
FRAAL5805		puf	3.65	I	+	+	+	−
FRAAL6852	*ssb*	Single-stranded binding protein 2	3.65	L	+	−	+	−
FRAAL4965	*gltB*	Glutamate synthase large subunit	3.65	EC	+	−	+	−
FRAAL0693		puf	3.60		−	−	−	−
FRAAL0937		Putative hydrolase	3.60	R	+	−	+	−
FRAAL4547		cpuf	3.55	S	+	+	−	−
FRAAL4964	*gltD*	Glutamate synthase small subunit	3.50	RE	+	−	+	−
FRAAL6658		puf; putative lysozyme-like domain	3.50		+	+	+	+
FRAAL3285		Putative aconitate hydratase B	3.50		−	−	−	−
FRAAL5052		puf	3.50		−	−	−	−
FRAAL5045	*bcp*	Peroxiredoxin	3.45	O	+	−	+	−
FRAAL5617		Putative AbaA-like regulator protein	3.45		+	−	+	−
FRAAL6283	*prs*	Phosphoribosylpyrophosphate synthetase	3.40	EF	+	−	+	−
FRAAL4052		Hypothetical membrane protein	3.35		−	−	−	−
FRAAL5804	*rnc*	Ribonuclease III	3.30	K	+	−	+	−
FRAAL5856	*gatC*	Glutamyl-tRNA amidotransferase subunit C	3.30	J	+	−	+	−
FRAAL5117		Putative Fur-family transcriptional regulator	3.20	PI	+	−	+	−
FRAAL5853	*gatB*	Aspartyl/glutamyl-tRNA amidotransferase subunit B	3.20	J	+	−	+	−
FRAAL1100	*rplO*	50S ribosomal subunit protein L15	3.10	J	+	−	+	−
FRAAL3297	*icd*	Isocitrate dehydrogenase	3.10	C	+	−	−	−
FRAAL5127		cpuf	3.10	S	+	−	+	−
FRAAL4376		Phosphofructokinase	3.05	G	+	+	+	−
FRAAL6146		puf	2.95		+	+	+	−
FRAAL6853	*rpsF*	30S ribosomal protein S6	2.95	J	+	−	+	−
FRAAL5053		puf	2.90		+	−	+	−
FRAAL6851	*rpsR*	30S ribosomal subunit protein S18	2.90	J	+	−	+	−
FRAAL5810		Putative RNA methylase	2.70	HL	+	−	+	−
FRAAL4586	*tpiA*	Triosephosphate isomerase	2.65	G	+	−	+	−
FRAAL5802	*mutM*	Formamidopyrimidine DNA glycosylase	2.65	L	+	−	+	−
FRAAL1926	*rpmA*	50S ribosomal protein L27	2.55	J	+	−	+	−

Table 75.1 (*Continued*)

Gene Number*	Gene Name*	Product Description*	Fold-Change†	COG ID‡	Core Genes§	Specific Genes**	Synteny††	Core Secretome‡‡
FRAAL1072	*rpoB*	DNA-directed RNA polymerase beta-chain	2.50	K	+	–	+	–
FRAAL6430		puf	2.50		–	–	–	–
FRAAL6266	*pth*	Peptidyl-tRNA hydrolase	2.40	J	+	–	+	–
FRAAL1424		Putative integral membrane export protein	2.35	RU	+	–	+	–
FRAAL0792		Putative high-affinity branched chain amino acid ABC transporter	2.35	E	–	–	+	–
FRAAL2883	*tatA*	Sec-independent twin-arginine translocase system protein	0.40	U	+	–	+	–
FRAAL3988		puf	0.40		–	–	–	–
FRAAL5491		Putative terpene synthesis protein	0.40		–	–	–	–
FRAAL0773	*aidB*	Acyl-CoA dehydrogenase (flavoprotein)	0.35	I	+	–	+	–
FRAAL1848		cpuf	0.35	V	+	–	+	–
FRAAL4345		puf	0.35		+	+	+	–
FRAAL5088		Putative esterase/lipase	0.35	R	+	–	+	–
FRAAL6201	*msrA*	Peptide methionine sulfoxide reductase	0.35	O	+	–	+	–
FRAAL1768		Putative CrP/Fnr-family transcriptional regulator	0.35	T	–	–	–	–
FRAAL1942		cpuf	0.35		–	–	–	–
FRAAL3379		puf	0.35	S	–	–	–	–
FRAAL6417		puf	0.35		–	–	–	–
FRAAL1053		Putative protease	0.30	O	+	–	+	–
FRAAL1304		Putative protein-glutamate methylesterase	0.30	TK	+	–	+	–
FRAAL0276		puf	0.30		–	–	–	–
FRAAL2771		puf	0.30		–	–	–	–
FRAAL3338		Putative carveol dehydrogenase	0.30	IQR	–	–	–	–
FRAAL5371		puf	0.30		–	–	–	–
FRAAL6326		puf	0.30		–	–	–	–
FRAAL2183		Putative membrane protein	0.30		+	+	+	–
FRAAL4301		Putative MutT-like protein	0.30	LR	+	–	+	–
FRAAL5412		Putative integral membrane protein	0.30		+	–	–	–
FRAAL5839		Putative CrP/Fnr-family transcriptional regulator	0.30	T	+	+	+	–
FRAAL6319		puf	0.30	K	+	+	–	–
FRAAL0138		puf	0.30	S	–	–	–	–
FRAAL1395		puf	0.30		–	–	–	–
FRAAL2607		cpuf	0.30		–	–	–	–
FRAAL3754		puf	0.30	S	–	–	–	–
FRAAL4380		Putative oxidoreductase	0.30	R	–	–	–	–
FRAAL5409		Glutamine amidotransferase class-II	0.30	MRE	–	–	–	–
FRAAL1024		puf	0.25		+	+	+	–
FRAAL1994		puf	0.25		+	+	+	–
FRAAL1995		Putative ferredoxin	0.25	C	+	–	–	–
FRAAL2005		Putative oxygen-independent coproporphyrinogen III oxidase	0.25	CH	+	–	+	–
FRAAL2149		Putative divalent cation tolerance protein	0.25	P	+	+	+	–
FRAAL2699		puf	0.25		+	–	+	–
FRAAL3771		cpuf	0.25		+	–	+	–

(*continued*)

Table 75.1 (*Continued*)

Gene Number*	Gene Name*	Product Description*	Fold-Change†	COG ID‡	Core Genes§	Specific Genes**	Synteny††	Core Secretome‡‡
FRAAL4206		Putative cytochrome P450	0.25	Q	+	−	+	−
FRAAL4947		puf	0.25	OP	+	+	+	−
FRAAL6525		Putative CarD-like transcriptional regulator	0.25	K	+	−	+	−
FRAAL2710		puf	0.25		−	−	−	−
FRAAL2813		puf	0.25	L	−	−	−	−
FRAAL3103		Putative phenylacetic acid degradation protein PaaA	0.25	S	−	−	−	−
FRAAL3131		puf	0.25	R	−	−	−	−
FRAAL3920		puf	0.25		−	−	−	−
FRAAL4218		Putative DNA topology modulation protein FlaR	0.25	FE	−	−	−	−
FRAAL5027		puf	0.25		−	−	−	−
FRAAL5161	*glnA*	Glutamine synthetase I (GSI)	0.20	E	+	−	+	−
FRAAL5664		Putative protein tyrosine phosphatase	0.20	T	+	+	+	−
FRAAL5886	*acs*	Acetyl-CoA synthetase	0.20	QI	+	−	+	−
FRAAL6021		Putative hydrolase	0.20	R	+	−	+	−
FRAAL6052		cpuf	0.20	SC	+	−	+	−
FRAAL0021		puf	0.20		−	−	−	−
FRAAL0216		Resolvase	0.20	L	−	−	−	−
FRAAL0533		cpuf	0.20	S	−	−	−	−
FRAAL1166		puf	0.20	TK	−	−	−	−
FRAAL1410		Putative oxidase	0.20	S	−	−	−	−
FRAAL3570		cpuf	0.20		−	−	−	−
FRAAL3953		Hypothetical membrane protein	0.20		−	−	−	−
FRAAL4027		puf	0.20	EM	−	−	−	−
FRAAL4028		puf	0.20		−	−	−	−
FRAAL4978		puf	0.20		−	−	−	−
FRAAL5356		Putative oxidoreductase	0.20		−	−	−	−
FRAAL0093		Putative RNA polymerase sigma factor	0.15	CK	+	+	+	−
FRAAL0517		cpuf	0.15		+	−	+	−
FRAAL1303		cpuf	0.15	J	+	−	+	−
FRAAL2313		Putative metallo-phosphos-hydrolase	0.15	R	+	−	−	−
FRAAL4243		Putative stress-inducible protein	0.15	T	+	+	−	−
FRAAL4321		Hypothetical glycine-rich protein	0.15	O	+	+	+	−
FRAAL6022		Putative aldehyde dehydrogenase	0.15	C	+	−	+	−
FRAAL6225		Putative ATP/GTP-binding protein	0.15		+	−	+	−
FRAAL0198		Putative transcriptional regulator	0.15	ROK	−	−	−	−
FRAAL0736		puf	0.15		−	−	−	−
FRAAL0891		puf	0.15		−	−	−	−
FRAAL0953		puf	0.15		−	−	−	−
FRAAL1394		puf	0.15		−	−	−	−
FRAAL1510		puf	0.15	S	−	−	−	−
FRAAL3031		cpuf	0.15		−	−	−	−
FRAAL4031	*hsp18*	Molecular chaperone Hsp18	0.15	O	−	−	−	−
FRAAL5103		puf	0.15		−	−	−	−
FRAAL5538		cpuf	0.15		−	−	−	−
FRAAL6787		cpuf	0.15		−	−	−	−
FRAAL0094		Putative serine protease, heat shock protein	0.10	O	+	−	+	+

Table 75.1 (*Continued*)

Gene Number*	Gene Name*	Product Description*	Fold-Change†	COG ID‡	Core Genes§	Specific Genes**	Synteny††	Core Secretome‡‡
FRAAL0629		Putative acetyltranferase	0.10	J	+	+	–	–
FRAAL2119		Putative integral membrane protein	0.10	OU	+	–	+	–
FRAAL2120		cpuf	0.10	O	+	–	+	–
FRAAL2150		puf	0.10	K	+	+	+	–
FRAAL2387		puf	0.10		+	+	–	–
FRAAL3148	*scoA*	Succinyl-CoA:3-ketoacid-coenzyme A transferase subunit A	0.10	I	+	–	+	–
FRAAL3149	*atoA*	Acetoacetyl-CoA transferase beta-subunit	0.10	I	+	–	+	–
FRAAL3311		cpuf	0.10		+	–	+	–
FRAAL3762		Putative sugar ABC transporter	0.10	EPG	+	–	–	–
FRAAL5832		puf	0.10	T	+	+	+	–
FRAAL5840		cpuf	0.10	R	+	–	+	–
FRAAL6548		Putative WhiB-family transcriptional regulator	0.10		+	–	+	–
FRAAL0140		Putative HTH-type transcriptional regulator	0.10	K	–	–	–	–
FRAAL0373		Putative acetyltransferase	0.10	R	–	–	–	–
FRAAL1344		puf	0.10		–	–	–	–
FRAAL2711		puf	0.10		–	–	–	–
FRAAL2947		puf	0.10		–	–	–	–
FRAAL3325		cpuf	0.10	VO	–	–	–	–
FRAAL4207		cpuf	0.10		–	–	–	–
FRAAL4467		Putative membrane protein	0.10		–	–	–	–
FRAAL4469		puf	0.10		–	–	–	–
FRAAL4664		puf	0.10		–	–	–	–
FRAAL6325		puf	0.10		–	–	–	–
FRAAL6418		puf	0.10		–	–	–	–
FRAAL0514		puf	0.05		+	+	+	–
FRAAL1014		puf	0.05	K	+	+	+	–
FRAAL3078		Transposase	0.05	L	+	–	+	–
FRAAL3310		cpuf	0.05		+	–	+	–
FRAAL6024		Putative glutamate-1-semialdehyde aminotransferase	0.05	EH	+	+	+	–
FRAAL6224		cpuf	0.05		+	–	+	–
FRAAL0300		Putative *s*-adenosylmethionine transferase	0.05	QR	–	–	–	–
FRAAL0515		puf	0.05		–	–	–	–
FRAAL0879		puf	0.05	Q	–	–	–	–
FRAAL1286		puf	0.05		–	–	–	–
FRAAL3763		Putative sugar ABC transporter	0.05	P	–	–	–	–
FRAAL4029		cpuf	0.05	QO	–	–	–	–
FRAAL4030		puf	0.05		–	–	–	–
FRAAL6577	*hppA*	Pyrophosphate-energized proton pump	0.00	CEGPR	+	–	+	–

(*continued*)

Table 75.1 (*Continued*)

Gene Number*	Gene Name*	Product Description*	Fold-Change†	COG ID‡	Core Genes§	Specific Genes**	Synteny††	Core Secretome‡‡
FRAAL0504		puf	0.00	R	–	–	–	–
FRAAL1946		puf	0.00		–	–	–	–
FRAAL3764		Putative sugar ABC transporter	0.00	G	–	–	–	–
FRAAL3765		Putative sugar ABC transporter	0.00	G	–	–	–	–
FRAAL5525		Putative acyl-CoA ligase	0.00	QI	–	–	–	–

ECF, extracytoplasmic function and EF-G, elongation factor G;

"+," present and "−," absent.

*Gene number, gene name, and product description according to the Genoscope database. puf stands for protein of unknown function, cpuf for conserved puf. Core genes are highlighted in gray.

†Fold-change comparing nodules versus FL cells, average of the two data sets.

‡Clusters of orthologous genes (COGs) classification ID (Tatusov et al. 2001).

§Core genes are defined as conserved genes in the three strains (ACN, EAN, and CcI3) at a threshold level of 40% amino acid identity over 80% of the length of the smaller gene.

**Specific genes are defined as core genes not conserved in *Acidothermus cellulolyticus* (NC_008578), *Streptomyces avermitilis* (NC_003155, NC_004719), and *S. coelicolor* (NC_003888, NC_003903, NC_003904) at a threshold level of 40% amino acid identity over 80% of the length of the smaller gene.

††Synteny is defined as the presence of a group of genes in the three strains (ACN, EAN, and CcI3), having 30% amino acid identity over 80% of the length of the smaller gene, separated by no more than five intervening sequences.

‡‡Core secretome encompasses 161 conserved secreted proteins in the three strains (ACN, EAN, and CcI3) according to Mastronunzio et al. (2008).

to the plant. *Frankia* in nitrogen-fixing pure culture can assimilate NH_4^+ produced by nitrogenase mainly through GSII a glutamine synthetase encoded by the *glnII* gene, whereas in N-replete culture, NH_4^+ is mainly assimilated by GSI, encoded by the *gln*A gene (Alloisio et al., 2010; Schultz and Benson, 1990). However, in symbiosis, the *glnA* gene is strongly downregulated (FRAAL5161, fc = 0.2), whereas the *glnII* gene (FRAAL5163) was not significantly induced, suggesting that symbiotic *Frankia* produces NH_4^+ that accumulates in vesicles and is then assimilated by the host plant enzymatic machinery.

In the *F. alni* genome, there are 426 genes labeled as involved in transport, export, or efflux, 229 of which are ATP-binding cassette (ABC) transporters. However, only about a dozen (or 5% of them) were differentially expressed, suggesting that nutrients and secondary metabolites exchanged between the two partners are reduced to the minimum, to focus bacterial activity onto nitrogen fixation. Finally, a gene (FRAAL0792) encoding a putative high-affinity branched-chain amino acid ATP-binding ABC transporter was 2.35-fold upregulated, permitting to suggest amino acids are also transported.

Concerning the uptake of plant photosynthates, the *Frankia* ACN *dctA* gene (FRAAL1390) was not differentially expressed and no *dct*A homolog is present in the CcI3 genome, suggesting another transporter could be involved. There are several poorly characterized ATP-binding ABC transporters (FRAAL2212, 2213, and 1843) that were two- to threefold upregulated but only in one of the two data sets. It is also possible that the reference for the arrays, which was *F. alni* growing on propionate in BAP, uses the same transporter, and thus, that there is no upregulation. Conversely, transporter genes exhibiting remarkable expression change were those coding for a putative sugar ABC transporter (FRAAL3762 to 3765), which were highly downregulated in nodules.

75.3.5 Comparison with Rhizobia Nodule Transcriptome

Rhizobia acquired a set of genes required for symbiotic nitrogen fixation via horizontal gene transfer (Sullivan and Ronson, 1998; see Chapter 21). Some rhizobia such as *Mesorhizobium* and *Bradyrhizobium* received them in the chromosome, and this structure is referred to as a symbiosis island (Kaneko et al., 2000, 2002). In the case of *Mesorhizobium loti*, genes within the symbiosis island are collectively expressed in nodule, forming an obvious "expression island" spanning about 600 kb (Uchiumi et al., 2004). In the case of *F. alni* nodule transcriptome, however, such a large island was not found and upregulated genes are scattered over the genome (see later), suggesting different origins of the symbiotic gene set between the two bacterial lineages.

Content of regulated genes and mode of their regulation in nodule are not similar between *Frankia* and rhizobia. *F. alni* and *Sinorhizobium meliloti* (Capela et al., 2006) shared 1179 orthologs, and 345 of them showed significant expression change in either of the bacteria. But only 16 of them were similarly upregulated (11) or downregulated (5) in nodule. Dominant genes upregulated in both bacteria were *nif*-related. Most of the upregulated genes in *F. alni* were downregulated or did not show significant expression

Table 75.2 Secondary metabolite gene clusters expression level

Gene Cluster	Genes	Metabolite Type	Proposed Product	Fold Change (Extreme Values)	Gene with Significant Fold Change
FA01	FRAAL0341–FRAAL0352	Type I modular PKS	Specialized lipid	0.84–1.61	None
FA02	FRAAL1275–FRAAL1282	Type I PKS	Mycocerosate-like lipids	1.01–2.02	None
FA03	FRAAL1549–FRAAL1558	Type I iterative PKS	Unknown	0.91–1.39	None
FA04	FRAAL1658–FRAAL1682	Type I iterative PKS	PUFA	0.58–5.35–10.80	FRAAL1682, 5.35
FA05	FRAAL1880	Quinone "NRPS"	Unknown quinones	0.91	None
FA08	FRAAL2558–FRAAL2576	Hybrid PKS-NRPS	Hybrid polyketide/peptide	0.39–1.14	None
FA10	FRAAL2909–FRAAL2914	Type I iterative PKS	Unknown, same as FA03	0.90–1.12	None
FA11	FRAAL2986–FRAAL2992	Type I PKS	Mycocerosate-like lipids	0.84–1.15	None
FA12	FRAAL3193–FRAAL3198	Type I iterative PKS	PUFA	0.93–1.15	None
FA14	FRAAL3421–FRAAL3473	Type I PKS	Beta-hydroxy butyrate	0.40–4.34	None
FA17	FRAAL4060–FRAAL4102	Type I PKS	Chlorothricin-like ring system	0.62–1.41	None
FA18	FRAAL4105	Quinone "NRPS"	Unknown quinones	1.15	None
FA19	FRAAL4152–FRAAL4172	NRPS	Siderophore	0.78–1.89	None
FA20	FRAAL4378–FRAAL4406	Type II PKS	Spore pigment	0.30–13.10	FRAAL4380, 0.30 FRAAL4403, 13.10
FA23	FRAAL6421–FRAAL6428	Non-NRPS siderophore	Siderophore, same as FC24, FE06	0.97–2.05	None
FA24	FRAAL6457–FRAAL6460	Type III PKS	Unknown, see also FE15	0.66–1.13	None
FA25	FRAAL2154–FRAAL2174	Terpene synthase	Carotenoids	0.41–1.33	None
FA26	FRAAL1427–FRAAL1449	Terpene synthase	Hopanoids	0.52–8.00	FRAAL1427, 8.00 FRAAL1430, 5.25 FRAAL1435, 6.90
FA27	FRAAL1335–FRAAL1339	Terpene cyclase	Pentalenene	0.90–1.31	None
FA28	FRAAL6507	Terpene cyclase	Geosmin	0.86	None
FA29	FRAAL6371–FRAAL6381	Phosphonate Unknown	Unk	0.84–1.27	None
FA30	FRAAL4919–FRAAL4922	Ribosomal peptide	Microcin-like	0.77–1.28	None
FA31	FRAAL4634–FRAAL4646	Aminocyclitol	Cetoniacytone-like	0.49–1.29	None

NRPS, nonribosomal peptide synthetase.

The list and cluster numbers are those proposed by Udwary et al. (2011). Highlighted in gray is the FA26 cluster for hopanoids synthesis.

changes in *S. meliloti*. Many of these genes were related to energy production (tricarboxylic acid cycle (TCA) cycle, respiration, and adenosine triphosphate (ATP) synthase) and translation (ribosomal proteins and translation factors). These results suggest that in the nodule, *Frankia* is metabolically more active than rhizobia. In contrast to rhizobia that are tamed by host plant being deprived of growth ability (Mergaert et al., 2003; see Chapter 67), *Frankia* appear to be more autonomous; for example, they have to synthesize vesicles to deal with lack of oxygen protection provided by host plants.

75.3.6 Localization on the Chromosome

Known symbiotic genes are not clustered on the genome, instead *nif* genes are close to the origin of replication, while the other clusters are scattered along the genome (Fig. 75.1).

75.4 CONCLUSION

No "smoking gun" upregulated genes could be identified that would have yielded new light on symbiosis mechanisms.

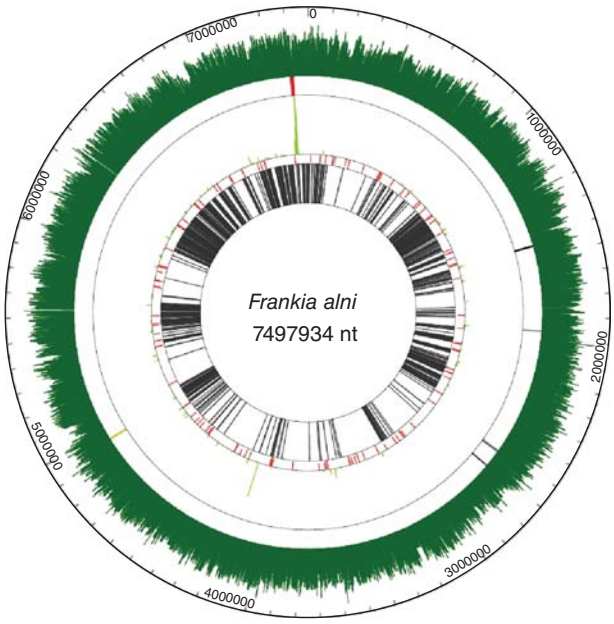

Figure 75.1 Circular representation of *Frankia alni* genome showing from the outside in (i) the GC% (in green), (ii) the symbiotic genes (*shc* genes in black, *hup* genes in gray, *suf* genes in yellow, *nif* genes in red), (iii) up-regulated genes (fc > 2, in light green), (iv) down-regulated genes (fc < 0.5, in red), and (v) conserved genome (>70% in aa).

Expected upregulated genes that were found to indicate symbiotic *Frankia* is essentially a nitrogenase machine. These upregulated clusters also indicate the DNA array approach functions but its dynamic range is probably too short, mainly because of the poor quality of high GC probes, to allow to identify symbiotic genes.

Deep sequencing of transcripts (RNA-seq) approaches should be used in the future to help identify the symbiotic determinants. The removal of ribosomal RNA is a critical step that still needs to be improved. RNA-seq can also help discern polycistronic messages, small regulatory RNAs, and tRNAs to obtain an enlarged view of the molecular regulation in symbiosis.

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Chapter 76

A Comprehensive Survey of Soil Rhizobiales Diversity Using High-Throughput DNA Sequencing

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76.1 INTRODUCTION

Numerous lineages from many bacterial phyla can fix nitrogen, but the majority of known rhizobia, nitrogen-fixing legume symbionts, are members of families (e.g., *Bradyrhizobia*, *Rhizobiaceae*, *Phyllobacteriaceae*) within the Rhizobiales, a diverse order within the bacterial subphylum Alphaproteobacteria.

In addition to nitrogen-fixing symbionts, the order includes lineages that are pathogenic to plants and animals and free-living lineages, some of which also fix nitrogen. A study comparing complete genomes from 30 Rhizobiales found that genomes of pathogens and symbionts contain both virulence and symbiotic genes and that horizontal gene transfer, especially of genes with activities related to host interactions and nitrogen fixation, contributes to the evolutionary plasticity of Rhizobiales (Carvalho et al., 2010).

In soil, Rhizobiales commonly fix nitrogen, and their abundance and community structure has been found to correlate positively with carbon-to-nitrogen ratio, the concentration of carbon, and the concentration of nitrogen (Shu et al., 2012; Will et al., 2010). In previously barren or disturbed soils, the abundance of Rhizobiales increases in response to new vegetation (Chen et al., 2012; Knelman et al., 2012). Agricultural practices can also affect Rhizobiales, and zero-tillage agriculture positively affects Rhizobiales abundance (Ceja-Navarro et al., 2010).

Environmental and site characteristics may affect specific lineages differently than they affect deeper taxonomic classifications. For example, abundance of the Acidobacteria phylum is negatively correlated with soil pH, but some subgroups within the phylum are positively correlated with pH (Jones et al., 2009). Similarly, environmental effects on rhizobia may differ from environmental effects on Rhizobiales as a whole. Although Rhizobiales are correlated positively with nitrogen content of the soil (Will et al., 2010), high levels of nitrogen limit diversity of *Rhizobium* populations (Caballero-Mellado and Martinez-Romero, 1999; Palmer and Young, 2000). Rhizobiales are much more abundant in the A horizon of soil (Will et al., 2010), but abundance of *Bradyrhizobium* under a leguminous tree does not differ substantially between surface and deep soils (Dupuy and Dreyfus, 1992).

A limitation of many studies on Rhizobiales diversity is the small number of soils analyzed and the focus on a specific genus or species. Until recently, surveying a large number of soils relied on fingerprinting-based approaches (e.g., denaturing gradient gel electrophoresis, terminal restriction fragment length polymorphism). While these approaches may be heuristically useful and necessary due to budgetary reasons,

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they are not optimal because the data generated are not linked to specific taxonomic groups and no phylogenetic or taxonomic information is generated (Jones et al., 2007). Fortunately, the development of barcoded primers has unleashed the power of high-throughput DNA sequencing and made it available to studies of microbial biogeography (Hamady et al., 2008). This approach works by attaching a unique barcode of 8–12 base pairs to the primer; DNA sequences generated contain that code which allows the sequence to be assigned to the correct sample. In this way, hundreds to thousands of samples can be analyzed in a single high-throughput DNA sequencing reaction.

The barcoded high-throughput approach has been used to explore biogeographic effects on soil bacterial and fungal communities with great success. A phylum-level analysis demonstrated a strong effect of soil pH on both bacterial diversity and composition (Lauber et al., 2009). In a comparison of bacterial and fungal diversity across an experimental pH gradient, pH strongly affected bacterial diversity but pH only weakly affected fungal diversity (Rousk et al., 2010). In a study of Arctic soils, pH again had the strongest effect on bacterial community structure and Arctic soils more closely resembled soils from across the globe with similar pH than Arctic soils with different pH (Chu et al., 2010).

These studies have improved our understanding of how ecological factors affect soil bacterial community structure and diversity. However, in general, these types of studies focus on deep taxonomic levels (e.g., all bacterial diversity), and the patterns of more fine-scale bacterial groups are ignored. In this chapter, a previously published high-throughput DNA sequence data set that characterized soil bacterial diversity from a large number of samples is considered but focused on diversity and community structure of Rhizobiales. In addition, two families within the Rhizobiales, the *Bradyrhizobiaceae* and the *Rhizobiaceae*, are also considered, because these families are commonly detected in soil and many lineages within them are symbiotic nitrogen fixers.

76.2 METHODS

A previous study used barcoded high-throughput DNA sequencing to generate bacterial 16S rRNA gene DNA sequences from 88 diverse soil samples collected across the Americas (Lauber et al., 2009). This study used 156,608 DNA sequences to examine general diversity patterns of soil bacteria, generally at the level of bacterial phyla, but did not focus on more fine-scale diversity patterns. Numerous environmental variables of the collection sites and soil samples were characterized, including depth of oxygen horizon, soil moisture, pH, carbon to nitrogen ratio, percentage of silt and clay, carbon mineralization rate, total organic carbon, total organic nitrogen, soil type, soil texture, latitude, longitude, elevation, annual precipitation, and mean annual temperature (Fierer and Jackson, 2006). Here, the DNA sequences and environmental data from this project are used to explore the factors associated with abundance and diversity patterns of Rhizobiales in soil.

DNA sequences were binned into phylotypes based on 97% DNA sequence identity using the uclust algorithm implemented in QIIME v1.6 (Caporaso et al., 2010). The most abundant sequence within each phylotype was designated the representative sequence, and these sequences were classified according to the Ribosomal Database Project's (RDPs; http://rdp.cme.msu.edu) classification scheme within QIIME.

The relative abundance of Rhizobiales within individual soil bacterial communities was compared to soil and site characteristics using Spearman's rank correlations. Similarly, the proportion of Rhizobiales classified as members of the *Bradyrhizobia* and *Rhizobiaceae* families was compared to soil and site characteristics using Spearman's rank correlations.

Beta-diversity patterns were analyzed to determine the effect of environmental factors on the community structure of Rhiziobiales. A rarefied data set was constructed, in which each soil sample was represented by 50 randomly chosen Rhizobiales sequences. Phylotype presence, absence, and abundance data from this rarefied data set were processed with the Bray–Curtis algorithm in QIIME to create a pairwise distance matrix of all soil Rhizobiales communities. Pairwise distances of environmental variables were constructed using Euclidean distances. The community and environmental matrices were compared using a mantel test in QIIME to determine if environmental variables significantly governed Rhizobiales community structure.

76.3 RESULTS

76.3.1 Rhizobiales Abundance and Diversity in Soils

Of 156,608 16S rRNA gene DNA sequences generated from the 88 soils, 14,388 (9.2%) were classified as Rhizobiales according to RDP classifications. The relative abundance of Rhizobiales in individual soils ranged from 3.7% to 18.2% across all soils, and the average relative abundance of Rhizobiales in soils was 9.2%.

Twelve families within the Rhizobiales order were detected in the soils (Table 76.1). Only two of these families, *Bradyrhizobiaceae* and *Hyphomicrobiaceae*, were detected in every soil sample and their proportion relative to the total amount of Rhizobiales detected ranged from 5.2–73.7% to 1.6–56.1%, respectively. The other frequently detected families included *Beijerinckiaceae*, *Methylobacteriaceae*, *Phyllobacteriaceae*, *Rhizobiaceae*, *Rhodobiaceae*, and *Xanthobacteraceae*.

Table 76.1 Summary of abundance and distribution of rhizobiales families across the 88 soil samples

Classification	#	Range (%)	Average Relative Abundance (%)	Standard Deviation (%)
Aurantimonadaceae	4	0–2.0	0.04	0.26
Bartonellaceae	2	0–0.8	0.02	0.11
Beijerinckiaceae	49	0–15.6	1.44	2.54
Bradyrhizobiaceae	88	5.2–73.7	31.53	15.48
Brucellaceae	1	0–1.8	0.02	0.19
Hyphomicrobiaceae	88	1.6–56.1	24.93	14.76
Methylobacteriaceae	35	0–19.5	1.22	3.01
Methylocystaceae	4	0–1.4	0.04	0.20
Phyllobacteriaceae	31	0–18.6	1.29	3.07
Rhizobiaceae	56	0–11.3	1.83	2.47
Rhodobiaceae	38	0–17.9	1.56	2.95
Xanthobacteraceae	27	0–3.1	0.31	0.64
Unclassified	88	17.0–73.3	35.76	9.51

Table 76.2 Spearman rank correlations between rhizobiales abundance and environmental variables

	R	p-Value
Carbon mineralization rate	0.411	<0.001
Annual precipitation	0.291	<0.01
Total organic carbon	0.278	<0.01
Moisture deficit	−0.268	<0.05
% Silt and clay	−0.239	<0.05
Carbon-to-nitrogen ratio	0.235	<0.05

Six of the measured environmental variables significantly correlated with Rhizobiales relative abundance (Table 76.2; Fig. 76.1). Carbon mineralization rate, annual precipitation, total organic carbon, and carbon-to-nitrogen ratio correlated positively with the relative abundance of Rhizobiales, whereas moisture deficit and % silt and clay correlated negatively with relative abundance of Rhizobiales.

Lineages within the *Bradyrhizobia* were commonly detected. The proportion of Rhizobiales classified as *Bradyrhizobia* ranged from 5.2% to 73.7% across the 88 soils. On average, 31.5% of Rhizobiales were classified as *Bradyrhizobia*. Five of the measured environmental variables significantly correlated with the relative abundance (relative to all Rhizobiales) of *Bradyrhizobia* (Table 76.3). Moisture deficit and pH correlated positively with relative abundance of *Bradyrhizobia*, whereas annual precipitation, total organic carbon, and % silt and clay correlated negatively with relative abundance of *Bradyrhizobia*.

Lineages within the *Rhizobiaceae* were detected in 56 (63.6%) of the soils. The maximum relative abundance of *Rhizobiaceae* (relative to all Rhizobiales) detected was 11.3%, and on average they represented 1.8% of all Rhizobiales in soil. The relative abundance of *Rhizobiaceae* significantly correlated with three measured environmental variables (Table 76.3). Relative abundance of *Rhizobiaceae* was positively correlated with pH and moisture deficit and was negatively correlated with elevation.

76.3.2 Distribution of Rhizobiales Phylotypes Across Soils

The 156,608 16S rRNA gene DNA sequences were binned into 47,283 phylotypes based on 97% DNA sequence similarity: 2052 (4.3%) phylotypes were classified as Rhizobiales. Of the Rhizobiales phylotypes, 1262 (61.5%) were detected in a single soil and 120 (5.8%) were detected in 10 or more soils. Some phylotypes were widely dispersed across a range of soils, but most were limited to only a few soil samples (Fig. 76.2).

The abundance and distribution of particular phylotypes was highly variable. Some phylotypes were not widely dispersed but were very abundant when present, some were widespread but rarely abundant, others were rare and never abundant, and one particular phylotype was widely dispersed and abundant (Fig. 76.3).

76.3.3 Factors Driving Rhizobiales Community Structure

Comparing Bray–Curtis community distances to Euclidean distances of environmental variables shows that eight environmental variables significantly affect Rhizobiales community structure (Table 76.4). Soil and site characteristics with significant effects on community structure include pH, followed by carbon-to-nitrogen ratio, total organic carbon, mean annual precipitation, carbon mineralization, latitude, temperature, and percentage of silt and clay (Table 76.4). Soil pH had the greatest effect on community composition (Fig. 76.4).

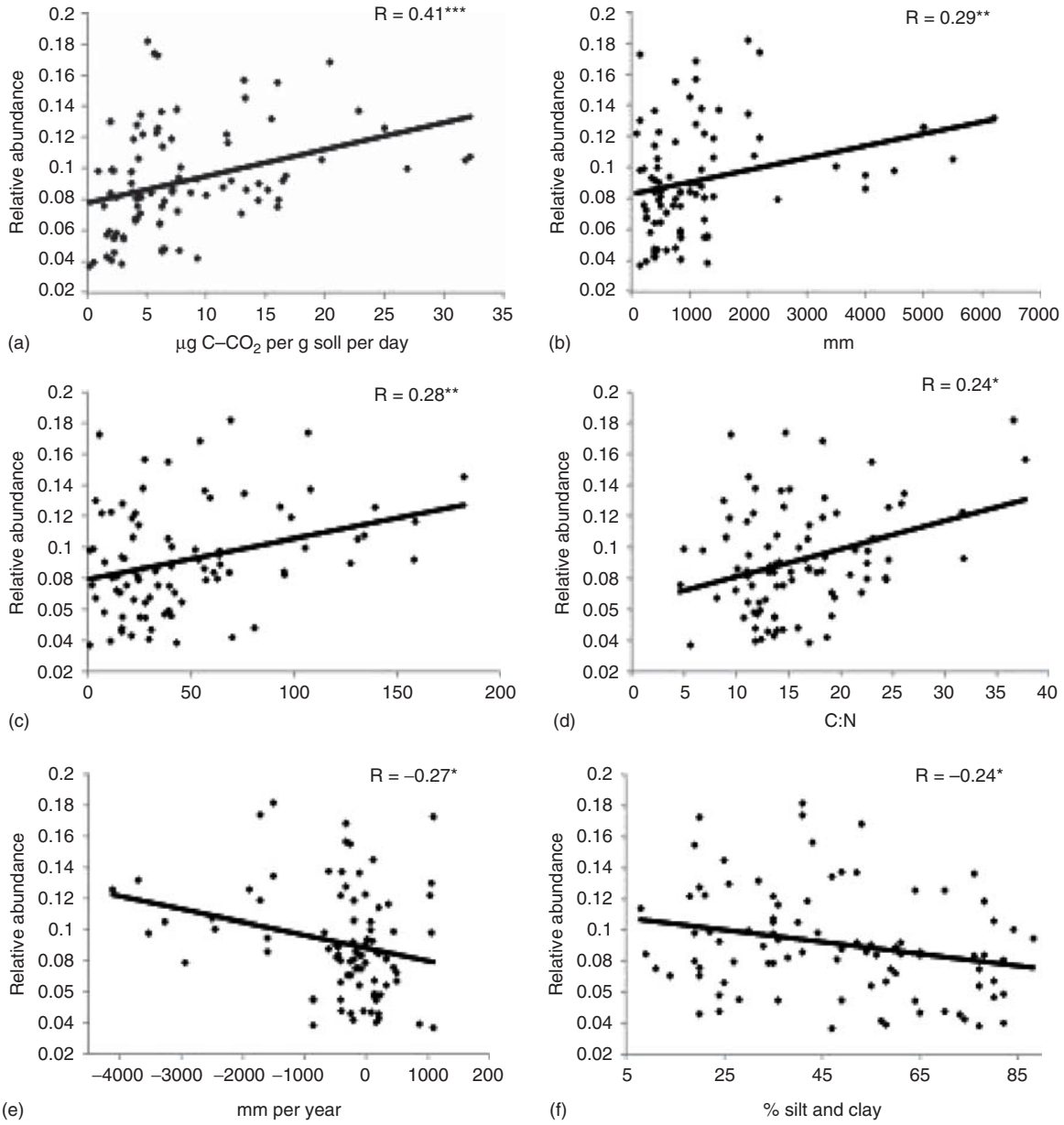

Figure 76.1 Soil and site characteristics with significant Spearman's rank correlations to relative abundance of Rhizobiales: potential carbon mineralization (a), annual precipitation (b), total organic carbon (c), carbon-to-nitrogen ratio (d), soil moisture deficit (e), and percentage of silt and clay (f). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

76.4 DISCUSSION

76.4.1 Factors Affecting Relative Abundance of Rhizobiales, *Rhizobiaceae*, and *Bradyrhiziobiaceae*

At the phylum level, soil pH strongly correlates with the relative abundance of some bacterial phyla such as, Acidobacteria, Actinobacteria, and Bacteroidetes, but not others such as Alphaproteobacteria and Beta-/Gammaproteo bacteria (Lauber et al., 2009). Since Rhizobiales is an order within the Alphaproteobacteria, it is not surprising that their abundance is not governed by soil pH. The relative abundance of soil Rhizobiales is most strongly correlated with potential carbon mineralization rates (Fig. 76.1, Table 76.2).

The relative abundances of families within the Rhizobiales do vary with pH, and the proportions of the two families that contain many nitrogen-fixing symbionts, *Bradyrhizobiaceae* and *Rhizobiaceae*, correlate positively

Table 76.3 Spearman rank correlations between bradyrhizobia and rhizobiaceae and environmental variables

	R	p-Value
Bradyrhizobia		
Annual precipitation	−0.490	<0.001
Total organic carbon	−0.453	<0.001
% Silt and clay	−0.408	<0.001
Moisture deficit	0.406	<0.001
pH	0.266	<0.05
Rhizobiaceae		
pH	0.315	<0.01
Elevation	−0.302	<0.01
Moisture deficit	0.280	<0.01

with soil pH. This finding is consistent with the well-known negative effects of soil acidity on rhizobial survival, multiplication, and nodulation (Graham, 1992).

76.4.2 Diversity Patterns of Rhizobiales

The distribution of Rhizobiales phylotypes across soils is similar to what is seen in other soil and marine systems. For example, a single acidobacterial phylotype was widely distributed across a range of soils and accounted for up to 7.4% of all detected bacteria (Jones et al., 2009). A bacterioplankton in the oceans, SAR11, is similarly widely distributed and oftentimes abundant (Morris et al., 2002]. This pattern of some lineages being both cosmopolitan and locally abundant was commonly detected in a meta-analysis of global patterns of microbial biodiversity (Nemergut et al., 2011). A Rhizobiales lineage in the 88 soils investigated here is also distributed among in a similar manner, in that it is widely distributed among soils and often abundant (Fig. 76.3). Another global pattern of microbial diversity is that the vast majority of phylotyopes are detected in a single sample (Nemergut et al., 2011). Again, soil Rhizobiales show a similar pattern with over 60% of phylotypes detected in a single soil.

76.4.3 Ecological Factors and Their Effect on Rhizobiales Community Structure

Although pH does not affect the relative abundance of Rhizobiales, it has the greatest effect on Rhizobiales community structure (Table 76.4); in other words, soil pH does not limit total abundance of Rhizobiales, but does control the presence or absence of certain Rhizobiales lineages. Using the same data set analyzed in this chapter, Lauber et al. found that pH strongly affected community structure of the full bacterial community and of the bacterial phyla Acidobacteria, Actinobacteria, Alphaproteobacteria, Beta-/Gammaproteobacteria, and Bacteroidetes (Lauber et al., 2009). Of all the other soil and site characteristics that Lauber et al. measured, only soil moisture deficit significantly correlated with bacterial community structure at the phyla level (Actinobacteria, Alphaproteobacteria, and Bacteroidetes). However, in Rhizobiales, carbon-to-nitrogen ratios, total organic carbon, mean annual precipitation, potential carbon mineralization rate, latitude, mean annual temperature, and % soil and clay also were significantly correlated with community structure (Table 76.4). These correlations were not strong (range from 0.121 to 0.243), but do indicate that at more fine-scale taxonomic resolutions,

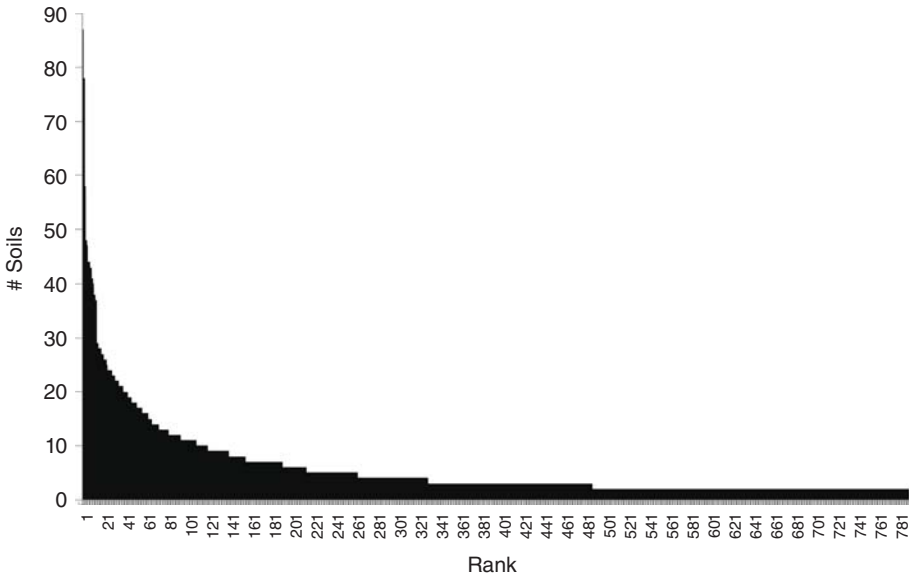

Figure 76.2 Distribution of Rhizobiales phylotypes among soil samples. One phylotype (rank 1) occurred in all 88 soils. Only 120 phylotypes occurred in 10 or more soil samples. The 1262 Rhizobiales phylotypes that occurred in only 1 soil were not included in the figure.

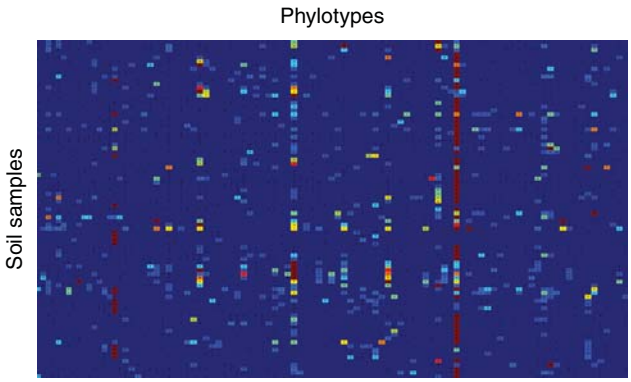

Figure 76.3 Heat map of the 100 most abundant (on average) Rhizobiales phylotypes across all 88 soils. Columns are Rhizobiales phylotypes and rows are soil samples.

Table 76.4 Mantel tests between microbial community Bray–Curtis distances and euclidean distances of environmental variables

Environmental Characteristics	Correlation	*p*-Value
pH	0.496	<0.001
Carbon-to-nitrogen ratio	0.243	<0.001
Total organic carbon	0.239	<0.001
Mean annual precipitation	0.232	<0.001
Carbon mineralization rate	0.208	<0.001
Latitude	0.193	<0.001
Temperature	0.140	<0.001
% Silt and clay	0.121	<0.001

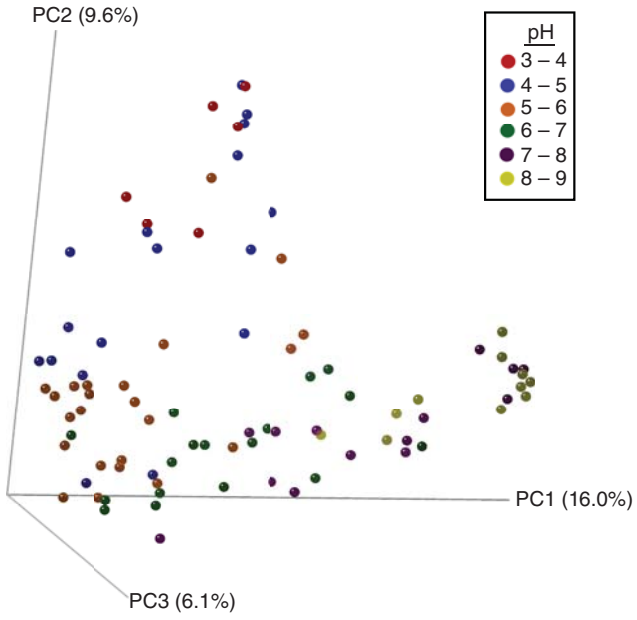

Figure 76.4 Three-dimensional principal component analysis of 88 soils based on Bray–Curtis distances. Each dot represents a Rhizobiales community and colors indicate the soil sample's pH.

these soil and site characteristics can have a greater influence on community composition.

76.5 CONCLUSION

Barcoded high-throughput DNA sequencing permits the characterization and comparison of a large number of microbial communities at a relatively inexpensive cost. This approach has been incredibly useful for determining how ecological factors govern microbial community dynamics, but they have generally focused on all bacterial diversity rather than on more fine-scale taxonomic groups. Here, it has been shown how this approach can also be useful for investigating microbial dynamics of shallower taxonomic groups, in this case the Rhizobiales.

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Chapter 77

Gene-Targeted Metagenomics of Diazotrophs in Coastal Saline Soil

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77.1 INTRODUCTION

Coastal land and the associated soil constitute a considerable portion of the biosphere. These lands are dynamic ecosystem, generally vulnerable to salinization, mainly due to high influx of tidal sea water. The habitat harbors taxonomically and functionally important halophiles and halotolerants as the selection pressure may favor the presence of microorganisms having physiological adaptations to survive under saline condition. The coastal habitat is unique in microbial diversity (Keshri et al., 2013b). The microbial community plays an essential role in the function and maintenance of the ecosystem through their profound effect on nutrient cycling. Nitrogen is one of the most essential nutrients for all life forms. Nitrogen fixation is a key element in the nitrogen cycling. The biogeochemistry of nitrogen on earth largely depends on the biological nitrogen fixation (Falkowski et al., 2008), contributing 61% of the total nitrogen added to the biosphere (Gruber and Galloway, 2008). Biological nitrogen fixation is mediated by free-living bacteria and symbiotic associations of phylogenetically diverse prokaryotic assemblages (proteobacteria, green sulfur bacteria, cyanobacteria, firmicutes, spirochaetes, and archaea). Nitrogen fixation is generally carried out by using the conserved enzyme complex nitrogenase reductase (Zehr et al., 2003; see Chapter 2). The detection and analysis of the marker gene(s), encoding nitrogenase reductase, provide an efficient tool to ascertain the biological nitrogen-fixation potential of an ecosystem. The Mo–Fe nitrogenase encoded by *nif* genes is the most efficient and widely distributed, while alternative nitrogenases, encoded by *vnf* and *anf* genes, are generally expressed only when molybdenum concentration is limiting (Zhao et al., 2006). The enzyme dinitrogenase reductase is encoded by the highly conserved *nifH* gene, which is being widely used to investigate diazotrophic diversity.

The culture-independent analysis of metagenomics has revolutionized the concept of microbial diversity, as the majority of microbes present in any habitat cannot be cultured. The community structure and diversity studies based only on rRNA analysis is insufficient for a complete understanding of the microbial ecology, as definitive information on biological, physiological, or ecological functions cannot be obtained (Zehr et al., 2003). Development of molecular techniques in recent years has allowed culture-independent detection of specific microbial populations, such as diazotrophs, by using the targeted analysis of the functional marker gene *nifH* (see Chapters 3, 8, 20). These methods involve polymerase chain reaction (PCR) amplification of *nifH* from metagenomic DNA, extracted from environmental samples, followed by clone library construction, denaturing gradient gel electrophoresis, restriction fragment polymorphism analysis, and/or quantitative real-time polymerase chain reaction (qRT-PCR/qPCR) to produce community profiles and phylogenetic relationship among diazotrophic microorganisms. It provides a comprehensive picture of the diazotrophic community structure and diversity compared to culture-based approaches. Molecular approaches have been used to investigate nitrogen fixation in a range of habitat by targeting nitrogenase genes to reveal a vast diversity of sequences. It leads to the enrichment of the database for nitrogenase genes (specifically the *nifH* gene; see Chapter 20), making it one of the largest nonribosomal gene data sets

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on uncultivated microbes (Zehr et al., 2003). However, until recently, little attention has been paid to the diazotrophic community inhabiting coastal saline soil.

The approaches and advantages of gene-targeted metagenomics have been reviewed by Iwai et al. (2011). Recently, the metagenomics of coastal soils have been investigated by targeting key functional genes (Keshri et al., 2013a, Yousuf et al., 2012a,b; Yousuf et al., 2014a,b). The gene-targeted metagenomics approach to study the diazotrophic diversity based on the phylogenetic analysis of *nifH* has been recently used in several other habitats including soils of different management practices (Colloff et al., 2008), maize field soil (Hsu and Buckley, 2009), rice cultivars (Wu et al., 2009), Arctic permafrost (Yergeau et al., 2010), Antarctic desert soil (Cowan et al., 2011), pristine shallow glaciofluvial ground water systems (Shirokova and Ferris, 2013), soils of different cropping systems (Pereira et al., 2011), Antarctic dry valley soils (Niederberger et al., 2012), the cactus rhizosphere (Aguirre-Garrido et al., 2012), rice root endophytes (Sessitsch et al., 2012), bulk soils of bioenergy crop field (Mao et al., 2013), and fresh water lake (Woodhouse et al., 2013). Others are focused specifically on saline system like deep sea methane vents (Pernthaler et al., 2008), hydrocarbon-polluted mangrove sediment (Taketani et al., 2009), deep sea methane seep sediments (Dang et al., 2009), rhizosphere of salt marsh plants (Gamble et al., 2010; Davis et al., 2011), Arctic sea water and sediments (Diez et al., 2012), Pacific warm pool (Shiozaki et al., 2013), sea sediments (Dang et al., 2013), and water column of Arabian Sea (Bird and Wyman, 2013). These studies revealed a large number of unknown sequences, which belong to diverse unidentified diazotrophs.

77.2 MATERIALS AND METHODS

77.2.1 Sample Size and Metagenome Extraction

Appropriate sample collection is a primary and essential step to achieve a comprehensive coverage of the microbial diversity. The coastal saline soil is generally barren and covered with salt deposition of different texture and concentration, devoid of regular vegetation but sometimes sparsely invaded by typical salt-tolerant plants. The soil samples were collected with a sterile scoop or spade in replicates and transferred to sterile polyethylene bags and stored at −20 °C during transportation. If the samples were rhizospheric, they were obtained from plants by collecting the soil around the roots and excising the young growing roots. To compare with nonrhizospheric diazotrophs, samples were taken from soil devoid of any plants. The respective subsample replicates were homogenized and mixed thoroughly and stored at −20 °C.

Gene-targeted metagenomics included the extraction of the soil metagenome and subsequent generation and screening of clone libraries, which were used for the exploration of complex functional groups of soil microbial communities. The validity of saline soil metagenomics was strongly dependent on an efficient extraction of high-quality microbial DNA to acquire the representative nucleic acids of the entire microbial communities, which is a technical challenge. The impediments included incomplete cell lysis, co-extraction of enzymatic inhibitors, degradation of DNA at various steps, uneven distribution of microbes within the soil matrix, and strong interaction of DNA as well as cells with the soil matrix (Grundmann, 2004; Rajendhran and Gunasekaran, 2008). Some recent reports have described a standard protocol for efficient extraction of the metagenome from coastal saline soil (Purohit and Singh, 2009; Siddhapura et al., 2010). The optimization of the total DNA extraction technique is critical and should be consistent with the sample types. The composition of habitats varies with respect to their matrix, organic/inorganic compounds, and biotic factors. The direct DNA extraction method via bead beating involves cell lysis within the sample matrix and subsequent separation of DNA from the matrix and cell debris (Ogram et al., 1987). This direct lysis method has been used recently for the construction of the *nifH* gene clone library from some of the saline soils such as the salt marsh plant rhizosphere (Gamble et al., 2010; Davis et al., 2011), mangrove sediment mesocosm (Taketani et al., 2009), sea sediment (Dang et al., 2013), and coastal saline soil (Keshri et al., 2013a,b; Yousuf et al., 2014a,b). Bead beating is one of the mechanical methods that allow good penetration of the lysis buffer by homogeneous cell disruption and sample dispersion. Therefore, it is more effective and less selective than the chemical lysis (Rajendhran and Gunasekaran, 2008). Siddhapura et al. (2010) obtained pure DNA in good quantity from saline soil of Gujarat, India, by using combinations of bead beating with lysis buffer treatment.

77.2.2 Primer Selection and PCR Amplification of the *nifH* Gene

The gene-targeted strategies for the study of diazotrophs require development of nitrogenase genes specific PCR primers. One of the widely used biomarkers is the *nifH* gene having advantages over other genes of the *nif* complex for investigating the diazotrophic assemblages. All of the dinitrogen reductase genes, namely, *nifH*, *vnfH*, and *anfH*, are highly conserved among diverse microorganisms, the phylogenetic tree based on the *nifH* gene is generally congruent with that of 16S rRNA gene (Ueda et al., 1995) and thus can be integrated with evolutionary analysis. The *nifH* gene database from environmental samples is very robust and has been comparatively well studied among

Table 77.1 Methods and primers used in gene-targeted diazotrophic analysis of saline samples

Samples	Methods	Primers		References
Mangrove sediments	RFLP clone library	nifHF: 5′-TCTACGGAAAGGGCGGTATCGG-3′ nifHR: 5′-GGCACGAAGTGGATCAGCTG-3′		Flores-Mireles et al. (2007)
Mangrove sediment	qRT-PCR, clone library	FGPH19: 5′-TACGGCAA(GA)GGTGG(TCGA)AT(TCA)G-3′ PolR: 5′-ATSGCCATCATYTCRCCGGA-3′	Simonet et al. (1991) Poly et al. (2001a)	Taketani et al. (2009)
Deep sea methane seep sediments	RFLP, clone library	nifHf: 5′-GGHAARGGHGGHATHGGNAARTC-3′ nifHr: 5′-GGCATNGCRAANCCVCCRCANAC-3′	Mehta et al. (2003)	Dang et al. (2009)
Bulk water of the Gulf	Clone library	nifH1: 5′-TGYGAYCCNAARGCNGA-3′ nifH2: 5′-DNGCCATCATYTCNCC-3′	Zehr and Turner (2001)	Foster et al. (2009)
Rhizosphere of the salt marsh cordgrass	DGGE	F: 5′-TACGG(P/K)AAKGG(P/G)GG(P/K)ATPGG-3′ R: 5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCG CCCCCGCCCG(G/C)ACG ATGTAGATPTCCTG-3′	Piceno et al. (1999)	Gamble et al. (2010)
Salt marsh plant rhizosphere	DGGE	F: 5′-TACGG(P/K)AAKGG(P/G)GG(P/K)ATPGG-3′ R: 5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCG CCCCCGCCCG(G/C)ACG ATGTAGATPTCCTG-3′	Piceno et al. (1999)	Davis et al., 2011
Water of hypoxic basins	Clone library qPCR	nifH1: 5′-TGYGAYCCNAARGCNGA-3′ nifH2: 5′-DNGCCATCATYTCNCC-3′ nifH3: 5′-ATRTTRTTNGCNGCRTA-3′ nifH4: 5′-TTYTAYGGNAARGGNGG-3′	Zehr and Turner (2001)	Hamersley et al. (2011)
		nifH3: 5′-ATRTTRTTNGCNGCRTA-3′ nifH4: 5′-TTYTAYGGNAARGGNGG-3′	Zani et al. (2000)	
		nif2F: 5′-TGAGACAGATAGCTATYTAYGGHAA-3′ nif623R: 5′-GATGTTCGCGCGGCACGAADTRNATSA-3′	Steward et al. (2004)	
Sea water, Sea ice brine, snow	DGGE, clone library	PolF: 5′-TGCGAYCCSAARGCBGACTC-3′ PolR: 5′-ATSGCCATCATYTCRCCGGA-3′	Poly et al. (2001a)	Diez et al. (2012)
		CNF: 5′-CGTAGGTTGCGACCCTAAGGCTGA-3′ CNR: 5′-GCATACATCGCCATCATTTCACC-3′	Olson et al. (1998)	
Oxygen-deficient waters of the Arabian Sea	Clone library	nifH4: 5′-TTYTAYGGNAARGGNGG-3′ nifH3: 5′-ATRTTRTTNGCNGCRTA-3′	Zani et al. (2000)	Jayakumar et al. (2012)
		nifH1: 5′ TGYGAYCCNAARGCNGA-3′ nifH2: 5′-ADNCGCATCATYTCNCC-3′	Zehr and McReynolds (1989)	

Table 77.1 (*Continued*)

Samples	Methods	Primers		References
Sea sediments	Clone library, qRT-PCR	nifHfw: 5′-GGHAARGGHGGHATHGGNAARTC-3′ nifHrv: 5′-GGCATNGCRAANCCVCCRCANAC-3′	Miyazaki et al. (2009)	Dang et al. (2013)
		nifHf: 5′-GGHAARGGHGGHATHGGNAARTC-3′ nifHr: 5′-GGCATNGCRAANCCVCCRCANAC-3′	Mehta et al. (2003)	
Upper water column of sea water	Clone library	nifH4: 5′-TTYTAYGGNAARGGNGG-3′ nifH3: 5′-ATRTTRTTNGCNGCRTA-3′	Zani et al. (2000)	Bird and Wyman (2013)
		nifH1: 5′ TGYGAYCCNAARGCNGA-3′ nifH2: 5′-ADNCGCATCATYTCNCC-3′	Zehr and McReynolds (1989)	
Surface water of Bay	Clone library	nifHf: 5′-GGHAARGGHGGHATHGGNAARTC-3′ nifHr: 5′-GGCATNGCRAANCCVCCRCANAC-3′	Mehta et al. (2003)	Gonzalez et al. (2012)
Coastal saline-alkaline soil	Clone library qPCR	PolF: 5′-TGCGAYCCSAARGCBGACTC-3′ PolR: 5′-ATSGCCATCATYTCRCCGGA-3′	Poly et al. (2001a)	Keshri et al. (2013a)
		nifH-rtF: 5′-CACCMCSATCAATCTGCT-3′ nifH-rtR: 5′-GCCATCATTTCGCCGGA-3′		

other genes of the *nif* complex (Zehr et al., 2003). The diazotrophs may belong to diverse group of proteobacteria, firmicutes, cyanobacteria, and euryarchaea (Raymond et al., 2004), exhibiting vast phylogenetic differences among nitrogen-fixers (Zehr and Mcreynolds, 1989). Therefore, designing universal *nifH* primers with a certain level of degeneracy is a prerequisite for broader analysis of diazotrophic diversity. A number of degenerate primers have been proposed by various researchers, but the degeneracy should be optimal for the coverage of maximum diversity with specificity. Degenerate primers are a mixture of primers with different binding affinities for different templates, which allow greater coverage of highly polymorphic genes (Wintzingerode et al., 1997). There are several primer sets available for the amplification of *nifH* gene commonly used for the saline habitat (**Table** 77.1). One of the most widely used primer set is PolF (5′-TGC GAY CCS AAR GCB GAC TC-3′) and PolR (5′-ATS GCC ATC ATY TCR CCG GA-3′), designed by Poly et al. (2001a) for environmental samples, and their efficiency were validated for different soil types (Poly et al., 2001b). The primer set contains sufficient degeneracy, 24 and 8 in PolF and PolR, respectively, which allows the amplification of a large number of diazotrophs from different phylogenetic groups. It selectively amplifies 360 bp of the *nifH* gene at an annealing temperature of 55 °C, which is used for meaningful derivation of phylogenetic relationship. The gene sequence database is increasing day by day; hence there is a quest for new primer data sets.

77.2.3 Molecular Analysis of the *nifH* Gene

Most of the gene-targeted studies of diazotrophs include PCR amplification of the *nifH* gene followed by clone library construction, restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE), and/or qRT-PCR analysis. Recently, DGGE was used to study the rhizospheric assemblages of diazotrophs associated with salt marsh cordgrass, *Spartina alterniflora* (Gamble et al., 2010). Analysis of DGGE banding pattern of the *nifH* gene amplified with GC-clamped primer pairs (Piceno et al., 1999) revealed diazotrophic assemblages associated with the saline system. The diazotrophs consisted of both seasonally responsive and nonresponsive groups. A study of the rhizospheric community from three different salt marsh plants, using DGGE, demonstrated that diazotrophs were strongly affected by seasonal changes and most of them were novel, indicating that diazotrophy is functionally redundant and widely performed by a large number of microorganisms (Davis et al., 2011). An Arctic marine ecosystem was recently investigated using these approaches to decipher total as well as cyanobacterial diazotrophic communities in sea water, sea brine, and snow (Diez et al., 2012).

Clone libraries and qRT-PCR were used to analyze the diversity and abundance of diazotrophs, respectively, in mangrove sediment mesocosms (artificial sea water fed tidal systems) (Taketani et al., 2009). The effect of oil addition on the diazotrophic community and the abundance in pristine and polluted mangrove sediments were also investigated, and the relationship between nitrogen fixation and hydrocarbon degradation was established (Taketani et al., 2009). Similar techniques were applied to study diazotrophic diversity and abundance in the water of hypoxic basins (Hamersely et al., 2011). The archaeal diazotrophic diversity was specifically characterized in methane seep sediments using the *nifH* gene clone library and qPCR approach (Miyazaki et al., 2009). The *nifH* gene clone library revealed the occurrence of noticeably distinct cluster of nitrogen-fixers in the surface water of a tropical bay (Gonzalez et al., 2012). The oxygen-deficient waters of the Arabian Sea were examined to explore the distribution, diversity, and expression of the *nifH* gene through analysis of a clone library, generated from DNA and cDNA (Jayakumar et al., 2012). Recently, the gene-targeted clone library of pelagic diazotrophs was coupled with reverse transcriptase PCR, and specific Northern slot blot analyses were performed to quantify *nifH* mRNAs and the active heterotrophic diazotrophs in the Arabian Sea (Bird and Wyman, 2013). The diazotrophic diversity and activity in the sea water of Gulf of Aqaba has been assessed using a clone library and reverse transcriptase PCR (Foster et al., 2009).

The RFLP and clone library techniques were used to reveal the diversity of diazotrophs from the deep sea methane seep sediments, which showed high degree of novelty in the *nifH* gene sequences with almost all of the sequences belonging to uncultured or uncharacterized bacteria or archaea (Dang et al., 2009). These techniques have been also used for diversity analysis from mangrove sediments from high and mid-intertidal zones. The biogeochemical characteristics of the rhizosphere determined the structure of the diazotrophs associated with the mangrove roots (Flores-Mireles et al., 2007). Dang et al. (2013) investigated different oligotrophic sediments including estuarine, coastal, offshore, deep-sea, and methane hydrate reservoirs or their prospective areas of South China Sea targeting the *nifH* gene using qRT-PCR and clone library construction, extending the evolutionary complexity of extant of the *nifH* gene with novel and diverse sequences.

77.2.4 Data Analysis and Diversity Estimation

Gene-targeted metagenomics require the extensive analysis of the *nifH* gene sequences and for which alignment of the obtained sequences is the primary step. This can be efficiently done by online tool CLUSTALW or by using software packages like MEGA 5. The similarity matrices

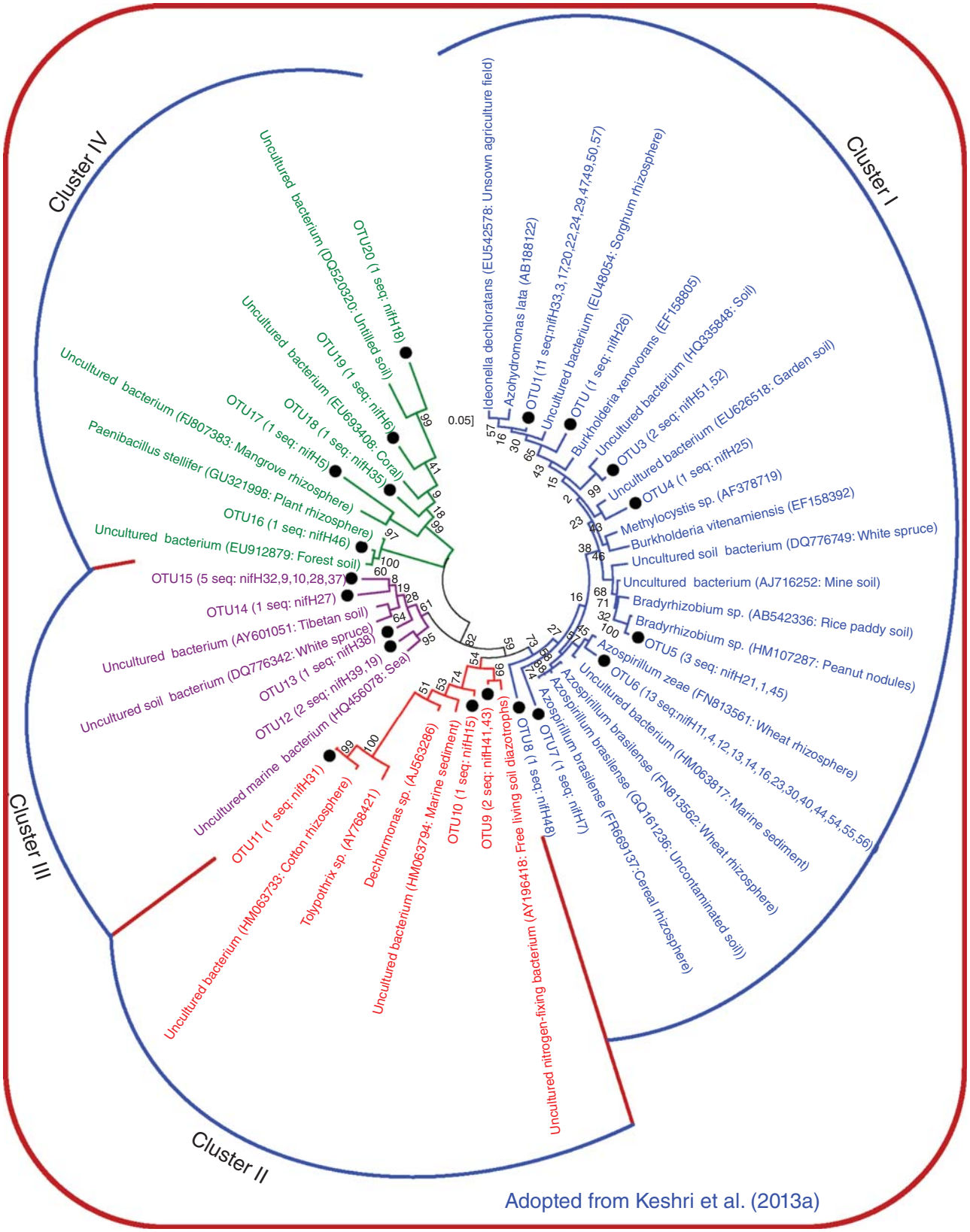

Figure 77.1 Molecular phylogeny of *nifH* using gene-targeted metagenomics of coastal saline-alkaline soil. The phylogeny was computed using the Tamura-Nei method and inferred with the maximum-likelihood method with the bootstrap test (1000 replicates), showed next to the branches. The tree was constructed with related sequences obtained from NCBI database, and in parentheses their accession numbers and source information are provided. OTUs from this study are provided after bullets (•).

generated from the aligned sequences are used to define operational taxonomic units (OTUs) using software (e.g., MOTHUR). There is no common consensus about the cutoff to define OTUs or the species for the *nifH* gene, like other functional genes. However, 90–97% sequence similarity was used to cluster the sequences into OTUs (Dang et al., 2009; Keshri et al., 2013a; Niederberger et al., 2012; Pereira et al., 2011; Taketani et al., 2009). A variety of statistical methods were used to estimate the diversity (Shannon, diversity index, Simpson index, species evenness, chao, ace) while multivariate analyses (hierarchical clustering, ordination plotting, canonical correspondence analysis, etc.) were used to reveal the pattern of diazotrophic community distribution (Aguirre-Garrido et al., 2012; Dang et al., 2009; Davis et al., 2011; Gamble et al., 2010; Niederberger et al., 2012). The community structure from the different samples is generally compared using the UNIFRAC while the genetic diversities are compared by the analysis of molecular variance.

77.3 A CASE STUDY: DIVERSITY AND ABUNDANCE OF *nifH* GENE IN COASTAL SALINE-ALKALINE SOIL

The ecological study of microbes from the coastal saline-alkaline soil is important in the context of *in situ* conservation of microbial diversity, which sustains delicate ecological processes in this type of ecosystem. We have conducted a study on the coastal soil having high salinity and alkalinity with EC 23.7 dS/m and pH 9.1 (Keshri et al., 2013a). The nitrogen-fixing community was targeted by analyzing the *nifH* gene using clone library and qPCR approaches, coupled with other functional genes involved in carbon fixation (*cbbL*), ammonia oxidation (*amoA*), and sulfur metabolism (*apsA*). The *nifH* gene was amplified using primers PolF and PolR (Poly et al., 2001a), and a clone library was constructed. The library contained 51 cloned sequences that categorized into 20 OTUs at the genetic distance of 0.1. About 25% library (13 clones) concurred with the alpha- and beta-proteobacterial species (*Bradyrhizobium* sp., *Azospirillum zeae*, *Azospirillum brasilense*, *Methylocystis* sp., *Burkholderia* sp., *Ideonella dechloratans,* and *Azohydromonas lata*). The *nifH* gene sequences representing cyanobacteria (*Tolypothrix* sp.-single clone) and Firmicutes (*Paenibacillus* stellifer-single clone) were also retrieved. These clones showed 91–97% similarity with the *nifH*-containing cultured species. However, 36 clones coincided with the environmental clones retrieved from various soil/sediment ecosystems such as rhizosphere, garden, forest, marine, the Tibetan plateau, and the Himalayas. These sequences formed four clusters in the phylogenetic tree (**Fig.** 77.1). The *A. lata*, *Bradyrhizobium* sp., and *A. zeae* were dominated in the cluster I. In this cluster, two OTUs (OTU7 and OTU8) showed a separate branching lineage without having any close affiliation and thus could be considered as novel. Cluster II and III sequences were not correlated with any known cultured organism and showed proximity with uncultured clones from the different environments. The Firmicutes, *P. stellifer* was the only cultured bacteria represented by clones from the cluster IV. While most of the sequences from the *nifH* gene clone library are not identical to those of the cultured nitrogen-fixing bacteria and the degree of divergence for most of the sequences obtained suggest the existence of novel nitrogen-fixing bacterial species in the selected soil type. The high degree of heterogeneity and low sequence similarity to the previously reported sequences suggested that the extreme physico-chemical conditions may influence the genetic structure of prevalent bacterial species and diazotrophs.

Abundance of the *nifH* gene determined by using qRT-PCR from the metagenome indicated the size of the functional guild harboring the relevant key gene for nitrogen fixation. The internal primers were designed from the conserved sequences of the clone library. 1.3×10^6 copies of the *nifH* gene per gram dry weight of soil were found, whereas the gene copies per gram dry weight of soil for the *cbbL*, *amoA,* and *apsA* genes were found to be $(1.64 \pm 0.42) \times 10^8$, $(1.19 \pm 0.21) \times 10^8$, and $(3.23 \pm 0.16) \times 10^5$, respectively. The abundance of the *nifH* gene was lower than the *amoA* gene, suggesting that oxidation of ammonia was more prevalent than nitrogen fixation. This study also suggested that the diversity and abundance of the functional genes are independent to each other and abundant gene may or may not be more diverse. The actual rate of the nitrogen fixation was not determined but the diversity and abundance of the *nifH* gene indicated the possible occurrence of the nitrogen-fixers in the hostile environment.

77.4 CONCLUSION

The study of diazotrophs based on gene-targeted metagenomics proved to be a highly efficient, accurate, rapid, and sensitive method to analyze nitrogen-fixing community present in the saline ecosystem. Our findings along with other observations demonstrated that we have still little knowledge about the microbes responsible for N_2 fixation in the coastal saline soil and the genetic diversity of N_2-fixers. The case study indicates that the saline habitats supported a unique diazotrophic community of diverse phylogenetic groups, suggesting distinctly different halotolerant nitrogenase diversity patterns in these stressful habitats. The gene-targeted metagenomic-based studies can be utilized for the isolation of novel indigenous diazotrophic species to be used as microbial inoculants for the agriculture practices in the coastal saline soils. The gene-targeted metagenomic from the coastal saline soils is indeed a valuable resource for hitherto untapped diazotrophs. Furthermore, the gene-targeted

metagenomics of diazotrophs could be explored to reveal the environmental factors controlling nitrogenase diversity.

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Section 14

Plant “Omics” and Functional Genetics

Chapter 78

The *Medicago truncatula* Genome

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78.1 INTRODUCTION

Due to their ability to efficiently fix atmospheric nitrogen in symbiotic association with rhizobia, legumes are of great environmental and agronomical importance, and for many years efforts have been made by the scientific community to better understand the biological process of symbiotic nitrogen fixation with the goal of eventually transferring the ability to fix nitrogen to nonlegume crops (see Chapters 5, 108, and 109). In parallel, research is carried out to enhance the attractivity of legume crops to farmers by improving yield, nutritional value, and resistance to biotic and abiotic stresses. Both approaches require the development of genetic and genomic resources for legume species. However, although early work on pea was instrumental in the development of genetics, overall legume crops are not very amenable to genetic and genomic approaches due to large genome size (pea, faba bean), polyploidy (soybean, alfalfa, clover), allogamy (alfalfa, clover), and difficult genetic transformation. Therefore, in the early 1990s, scientists selected *Medicago truncatula* as a model legume due to favorable attributes such as annual habit, short life cycle, abundant seed production, diploid and autogamous nature, small genome size (around 550 Mbp), ease of genetic transformation by *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*, and nodulation by the well-characterized *Sinorhizobium meliloti* rhizobial bacterium (Barker et al., 1990; Cook, 1999). In addition, contrary to the model plant *Arabidopsis thaliana*, *M. truncatula* can establish symbioses with endomycorrhizal fungi that are important for plant mineral nutrition and whose establishment shares mechanisms with nitrogen-fixing symbioses. *Lotus japonicus*, another model legume, has similar features but *M. truncatula* is phylogenetically closer to temperate legume crops such as alfalfa, clover, pea, or faba bean, making it a better choice for translational genomics.

78.2 A REFERENCE ANNOTATED GENOME SEQUENCE FOR *Medicago truncatula* A17

The availability of a reference genome sequence for *M. truncatula* was central to the development of functional genomic resources in this species, the analysis of its natural diversity and evolution, as well as for comparative genomic studies in legumes. *M. truncatula* genome sequencing was thus started in 2002. At that time, *A. thaliana* was the only plant with a sequenced genome, and whole genome shotgun sequencing was not considered a realistic strategy to provide a high-quality reference genomic sequence anchored to chromosomes, for a genome of more than 500 Mbp like that of *M. truncatula*. It was thus decided to sequence *M. truncatula* by the same strategy than that previously chosen for the *A. thaliana* and rice genomes: sequencing the genome clone by clone (BAC by BAC) (bacterial artificial chromosome), each clone being anchored to chromosomes through physical and genetic mapping (Young et al., 2005). Similar strategies were adopted at the time for the genomes of plants such as *L. japonicus* (Sato et al., 2008) or tomato (Sato et al., 2012). This approach allowed sharing the work chromosome by chromosome between different groups, and an international consortium with American and European partners was established to sequence the *M. truncatula*

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genome. In particular, due to the high costs of the Sanger sequencing technology then available, it was decided to focus the sequencing to the gene-rich part of the genome. Previous cytogenetic analysis using fluorescence *in situ* hybridization (FISH) on pachytene chromosomes indicated that in *M. truncatula* most chromosomes could be divided into euchromatic arms, rich in genes, and heterochromatic pericentromeric regions rich in repeated elements (Kulikova et al., 2001): about a hundred gene-rich BACs tested hybridized only to chromosome arms. Large-scale sequencing thus started with 500 gene-rich (expressed sequence tag (EST) containing) nonoverlapping seed BACs. These BACs were anchored to chromosomes by genetic mapping using microsatellite markers identified in their sequences (Mun et al., 2006). BAC by BAC sequencing then proceeded by a sequence tag connector approach: from a sequenced seed BAC, two BACs with minimum overlap with the seed BAC were selected on each side based on alignment of their BAC end sequences to the seed BAC sequence, then sequenced. The same process was repeated for every newly sequenced BAC, leading to large BAC contig sequences that eventually merged. Selection of BACs was also guided by a physical map constructed by restriction enzyme fingerprinting, and the progression of the assembly checked by further genetic mapping (Young et al., 2011). The final assembly was validated by optical mapping. This approach was pursued to its term in the case of chromosome 5, resulting in two sequence contigs corresponding to the two chromosome arms, covering 42 Mbp of sequence from telomeres to pericentromeric region. Due to lack of funding, BAC by BAC sequencing of the other chromosomes could not be completed. Thus, the 262 Mbp final assembly of BAC sequences included many gaps in chromosome arms and missed about one-third of *M. truncatula* genes. Further sequencing was performed using next-generation technologies, mainly Illumina. Assembly of sequences obtained from paired end libraries resulted in 100 Mbp of additional sequence contigs with N50 = 2.3 kb, allowing characterization of about 20% more genes without anchoring to chromosomes (Young et al., 2011). In the past 2 years, next-generation sequencing, using in particular paired end and mate pair Illumina technology, was used to sequence DNA libraries carrying inserts of various sizes (from a few hundred bp to more than 10 kbp). These new resources, together with data previously obtained by the consortium (BAC end sequences, genetic and optical maps, etc.) and improved bioinformatic tools, led to the development of new genome assemblies anchoring to chromosomes more than 80% of the genome and covering 95% of *M. truncatula* genes (Debellé et al., 2012; Krishnakumar et al., 2013). The sequence assembly covers most of the euchromatin (the chromosome arms), while the pericentromeric heterochromatin remains unsequenced.

Gene annotation of the genome assembly was carried out by an annotation consortium using the Eugene pipeline, which combines the use of *ab initio* predictions to that of extrinsic data (cDNA/EST, RNA-seq, homologies to gene sets from other genomes) (Town, 2006; Young et al., 2011). The number of predicted genes varied according to the level of confidence of prediction from less than 50,000 to more than 60,000 protein-coding genes. This is a high number compared to that of the related chickpea (28,000 genes), and it is similar to the number found in soybean, although this species, contrary to *M. truncatula*, is known to have undergone a recent (14 Myr ago) whole genome duplication. This high number can be explained at least, in part, by high levels of local gene duplication in *M. truncatula*, which affect many gene families and are detected across the genome (Young et al., 2011).

78.3 *M. truncatula* GENOME ORGANIZATION AND EVOLUTION

Although BAC by BAC sequencing excluded the pericentromeric regions of *M. truncatula* genome, early analysis of whole genome shotgun sequences had allowed the identification of tandem repeats with head-to-tail orientation, with unit lengths varying from 166 to 183 bp, typical of satellite repeats found in the pericentromeric regions in various plant species (Kulikova et al., 2004). Three of them, accounting for 1–4% of the genome, were characterized by FISH on pachytene chromosomes (Kulikova et al., 2004). MtR3 was found in centromeric regions (visible as primary constrictions) of all chromosomes, while MtR1 and MtR2 were located in distinct pericentromeric regions of most chromosomes. FISH also showed the presence of 5S rDNA loci on chromosomes 2, 5, and 6 and of 45S rDNA genes on chromosome 5 (Kulikova et al., 2004). The pericentromeric regions are likely to contain, in addition to tandem repeats, a large number of transposable elements, in particular retrotransposons, as is the case in many plant genomes (Du et al., 2010). Transposons have been characterized in detail in the sequenced part of the genome where they constitute about 30% of the sequence (Young et al., 2011). While DNA transposons constitute 3–4% of the sequence, long terminal repeat retrotransposons are the most abundant transposable elements (24% of the sequence) with copia and gypsy families, representing 4.1 and 5.7% of the sequenced genome, respectively (Young et al., 2011). Different families of retrotransposons, also present in other plant genomes in different proportions, have been characterized (Du et al., 2010; Wang and Liu, 2008). Along chromosomes, the density in retrotransposons varies, with possibly an increase toward the pericentromeric regions.

Self-comparison of the *M. truncatula* genome revealed conserved synteny blocks reflecting duplication events (Cannon et al., 2006). The rates of synonymous substitution per synonymous site (Ks) between paralogs in these

blocks indicated that they were the remnants of a whole genome duplication event that occurred ~58 Myr ago, early in the evolution of the Papilionoideae subfamily of legumes. Comparative genome analysis in the legume family and other higher plants also provided evidence for this duplication and clues about legume evolution (Young and Bharti, 2012; Young et al., 2011). All legumes and major groups of dicots share an ancient whole genome hexaploidy anterior to the rosid–asterid split at ~150 Myr ago. The whole genome duplication event at 58 Myr ago affected the Papilionoideae subfamily but apparently not the Mimosoideae and Caesalpinioideae subfamilies: transcriptome analysis of the mimosoid *Chamaecrista* provided no evidence of the 58 Myr whole genome duplication (Cannon et al., 2010); however, further work is required to characterize more genomes in the nonpapilionoid legumes. After the whole genome duplication, the Papilionoideae soon radiated into several clades. Among them, the millettioids (*Glycine*, *Phaseolus*, *Cajanus*) and the galegoids (*Medicago*, *Pisum*, *Lotus*, *Cicer*) separated ~54 Myr ago. *Medicago* diverged from *Lotus* ~50 Myr ago and from *Cicer* ~10–20 Myr ago. A more recent whole genome duplication occurred in soybean about 14 Myr ago (Young and Bharti, 2012; Varshney et al., 2013). Comparison of sequenced legume genomes extended previous genetic map-based comparative analyses and revealed extensive conservation of synteny, extending to whole chromosome arms (Choi et al., 2004a,b; Varshney et al., 2012, 2013; Young and Bharti, 2012). Comparison of synteny blocks conserved between different legume species allows reconstruction of an ancestral legume karyotype (Salse, 2012). There are few remnants of the 58 Myr whole genome duplication in the *M. truncatula* genome probably due to intense gene fractionation after the duplication event. However, subfunctionalization and neofunctionalization of homeologs can occur after duplication, and these processes could have played a role in the evolution of rhizobial nodulation (Young et al., 2011).

78.4 NATURAL VARIATION IN *Medicago* GENOMES

The availability of a reference sequenced genome opens the way to an in-depth analysis of natural variation within the *M. truncatula* species. The decreasing costs of sequencing make resequencing (alignment of short sequence reads on a reference genome sequence for the discovery of single-nucleotide polymorphisms (SNPs) of a large number of accessions affordable. A large number of *M. truncatula* accessions from around the Mediterranean basin have been collected (http://www1.montpellier.inra.fr/BRC-MTR/accueil.php), and the structures of these populations have been studied using a limited number of markers such as microsatellites (Ronfort et al., 2006). This allowed establishing core collections representative of the species diversity. In the *M. truncatula* hapmap program, 26 *M. truncatula* accessions from core collections were sequenced at 30× coverage, 226 accessions at 8× coverage (Branca et al., 2011; Stanton-Geddes et al., 2013). This allowed the identification of more than 6 million bi-allelic SNPs (about 10 sequence variants every 1 kb). These data were used to characterize genome-wide patterns of diversity, recombination rates, and linkage disequilibrium (Branca et al., 2011). Nucleotide diversity was found to be higher than in soybean or *Arabidopsis*. It decreased from centromeric to telomeric chromosomal regions and was negatively correlated with gene density, suggesting diversity is shaped by selection against deleterious mutations (Branca et al., 2011; Paape et al., 2013). Linkage disequilibrium was found to decay to half of the initial value within 3–4 kb, similar to what is described for *A. thaliana* but was very variable in different chromosomal regions. The population recombination rate was approximately one-third of the mutation rate, compatible with the high selfing rate of *M. truncatula* (Branca et al., 2011). Intraspecies diversity and interspecies divergence with *M. sativa* was also investigated using sequence data for >20,000 annotated genes (Paape et al., 2013): 50–75% of nonsynonymous polymorphisms were found under strong purifying selection, and only 1% of genes showed a signature of putative positive selection, among them genes involved in symbiotic and pathogenic interactions as well as epigenetic modifications. Whole genome interspecies comparison within the *Medicago* genus also helped clarify phylogenetic relationships between different *Medicago* species (Yoder et al., 2013). Finally, resequencing of *M. truncatula* lines opens the way to genome-wide association studies: statistical associations between sequence polymorphisms and phenotypic variation among natural accessions enable the identification and localization of putative causative loci. Such an analysis was conducted in *M. truncatula* to explore the genetic architecture of complex traits (flowering time, height, nodulation) and identify candidate genes underlying these traits (Stanton-Geddes et al., 2013). A number of other studies dealing with resistance to pathogens or symbiotic interactions with rhizobia or mycorrhizal fungi are in progress.

78.5 THE *M. truncatula* GENOME AND FUNCTIONAL GENOMICS

78.5.1 Transcriptomics

Many plant physiological and developmental processes are controlled at the transcription level, and the differential expression of genes in specific organs/conditions can give clues about their functions. Transcriptome analyses can be conducted in the absence of a complete reference

genomic sequence, for example, by sequencing ESTs or RNA sequencing. However, the availability of a genomic sequence, as is the case for *M. truncatula*, greatly benefits those analyses. EST data bases are often incomplete due to the low level or very specific expression of a number of genes. The gene models in the annotated *M. truncatula* genome sequence were thus used, in addition to EST sequences, to define the probes in microarrays used for transcriptome analysis, resulting in more comprehensive and specific sets of probes (Benedito et al., 2008). Over the years, more and more complete arrays have been made available, and the recent improvements in the genome sequence and its annotation should translate into still better arrays for transcriptomic analysis of protein-coding genes and open the way to tiling arrays. More recently, direct RNA sequencing approaches have been used to characterize the transcriptome of *M. truncatula*, and the bioinformatic analysis of RNA seq data generally involves mapping of the RNA sequences to the reference genomic sequence before the quantification of gene expression (Boscari et al., 2013; Young et al., 2011). It is worth noting that transcriptome data are precious to improve the quality of the genome annotation, allowing a better characterization of gene models and identification of alternative splicing sites (Town 2006; Cheung et al., 2006). A large number of transcriptomic studies have been carried out in *M. truncatula*, to identify genes differentially expressed in specific organs, in response to biotic and abiotic stresses, in the course of developmental processes (seed development, nodule development, etc.) (see Chapters 79 by Valdes-Lopez and 82 by Sinharoy et al.). It is in the field of symbiotic nitrogen fixation that these approaches have been most developed, with refinements including the use of developmental time series, of laser capture microdissection of specific nodule zones, and of various bacterial and plant mutants affected in the symbiotic process and treatments by symbiotic signal (Limpens et al., 2013; Moreau et al., 2011; Murray et al., 2011; Roux et al., 2014). These efforts have led to the identification of genes differentially expressed during the establishment of the symbiosis, among them transcription factors and other regulators that are good candidates for further functional characterization, in particular by reverse genetics approaches.

78.5.2 Proteomics

Proteomics is an important research tool to analyze biological processes. It can provide clues about protein localization, protein–protein, or protein–ligand interactions, posttranscriptional modifications, etc. Proteomic analysis generally involves high-resolution protein purification followed by proteolytic digestion and mass spectrometry (MS) and can lead to protein identification and quantification. MS data are used to search predicted proteins from DNA, RNA, or sequenced protein databases. The quality of the *M. truncatula* genome assembly and annotation is thus a crucial parameter for the success of proteomic analysis in this species. In addition MS analysis of *M. truncatula's* proteome could be used to improve the structural annotation of its genome in a proteogenomic approach (Volkening et al., 2012). Many proteomic studies of *M. truncatula* have been published, several of them dealing with the analysis of symbiotic interactions (e.g., Fliegmann et al., 2013; see Chapter 51), and also that of seed development or the proteomes of specific organs, tissues, or subcellular compartments (Lee et al., 2013). Phosphoproteome analyses were also performed to characterize components of symbiotic signaling since this process is known to involve several protein kinases (Rose et al., 2012).

78.5.3 Small RNA Analysis in *Medicago*

The availability of a reference genome for *M. truncatula* was crucial for the analysis of small noncoding RNAs in this species. Their known role in plant development and responses to biotic and abiotic stress suggested a potential role in nodulation and more generally in symbiotic interactions between legumes and rhizobia, or mycorrhizal fungi, and early on they were the focus of several analyses (e.g., Bustos-Sanmamed et al., 2013). Small noncoding RNAs, generally 20–24 nucleotides long, are generally classified into two groups, microRNAs (miRNAs) and small interfering RNAs (siRNAs), that have different biogenesis pathways and mechanisms of gene regulation (Axtell, 2013).

The *M. truncatula* genome contains six genes encoding members of the four groups of DCL (Dicer-like) proteins characterized in *Arabidopsis* and other plants that are required to cleave smRNAs precursors (including three DCL2-like proteins). Sixteen genes in the *M. truncatula* genome, in four main clades, encode AGO (Argonaute) proteins, which bind to miRNAs and participate in silencing effector complexes (RNA-induced silencing complex, RISC). Compared to *Arabidopsis* these two families have expanded, but it is not known whether this led to specialization in expression or function. More work using mutants in the different genes or coimmunoprecipitation of AGO proteins could shed light on the respective roles of DCL or AGO family members in the biosynthesis and modes of action of smRNAs (Bustos-Sanmamed et al., 2013).

miRNAs are by far the most studied smRNAs in *M. truncatula*. These smRNAs, often 21 nt long, derive from single-stranded RNA precursors that have a stem-loop secondary structure. DCL1 proteins recognize the secondary structure and generate an miRNA–miRNA* duplex. From the duplex, the miRNA binds to an AGO protein in an RISC, where the miRNA pairs to a complementary sequence in a target mRNA, leading to target cleavage or posttranscriptional repression (Axtell, 2013). The first miRNA in

M. truncatula was identified based on their sequence similarities to miRNA characterized previously in *Arabidopsis* (Boualem et al., 2008; Combier et al., 2006). More recently, the combination of high-throughput sequencing of smRNA libraries and better genomic resources allowed identification of miRNA in *M. truncatula* (Lelandais-Briere et al., 2009; Young et al., 2011). A high-quality reference sequence is required since a number of filtering steps, including mapping to the reference genome, elimination of structural RNAs and repeats, prediction of precursors based on miRNA:miRNA* pairing evaluation, and stem-loop structure are applied to smRNA sequences before the smRNAs can be considered *bona fide* miRNA candidates (Meyers et al., 2008). The miRNA targets were predicted based on the sequence similarity between miRNA and their targets, and in several cases the predictions were validated by degradome/PARE (parallel analysis of RNA ends) experiments, which identify the cleavage site in putative miRNA targets (Devers et al., 2011; Zhai et al., 2011). The current version of miRBase lists 756 mature miRNAs, a high number compared to other plants. This list is likely to evolve since all predictions were performed on incomplete versions of the reference genome, and new smRNA libraries are regularly sequenced. On the other hand, more stringent criteria for miRNA validation might remove some of them from the database. Part of the miRNA belong to families conserved between multiple plant species while some are more lineage specific (Bustos-Sanmamed et al., 2013).

Among siRNAs, tasiRNAs (trans-acting small interfering RNAs) are generated from transcripts that are first cleaved through the action of 22 nt miRNA triggers, and then transcribed into long dsRNAs that are processed by DCL4 into 21 nt tasi RNAs in 21 nt phased intervals (called phasiRNAs). More than 110 PHAS (phasiRNA-producing) loci were identified in *M. truncatula*, the majority being genes encoding defense-related NBS-LRRs (Fei et al., 2013; Zhai et al., 2011) (see below).

Finally, a major class of siRNA is heterochromatic siRNAs, mostly 23–24 long, which are associated with repressive chromatin modifications acting on the activities of transposable elements. Those have not been much studied in *M. truncatula*. Their analysis would benefit from a better characterization of heterochromatic regions of the *M. truncatula* genome and annotation of repeats.

78.5.4 Reverse and Forward Genetics

The genes identified by the above-described -omics approaches can be further tested functionally through inactivation using a reverse genetics methodology. RNA interference has been used successfully to functionally characterize several symbiotic genes, but the technology was never used on a large scale. The most efficient resource for gene inactivation was developed in recent years by European and US scientists who implemented insertion mutagenesis using the tobacco retrotransposon Tnt1. More than 20,000 insertion lines have been generated with probably overall more than 500,000 insertions in the genome (Cheng et al., 2011) (see chapters by Pislariu et al., Sinharoy et al.; see Chapters 82, 83) often resulting in knock-out mutations in genes. Mutations in genes of interest can be screened through a polymerase chain reaction (PCR) platform at the Noble Foundation or directly using Tnt1-insertions flanking sequences available in a regularly updated database. It is worth noting that most of the Tnt1 insertions have been generated in the R108 line of *M. truncatula*, which is transformed more easily than the A17 line. Although A17 and R108 are closely related, there are significant differences in their genome sequences. A *de novo* sequencing of the R108 genome with the current high-throughput technologies used to complete the A17 genome sequence would thus certainly be helpful to improve screening of the Tnt1 insertion lines. Several TILLING (targeting-induced local lesions in genomes) populations have also been developed for *M. truncatula* reverse genetics (Le Signor et al., 2009). They are very useful to obtain allelic series of point mutations in genes of interest for detailed structure function analyses, but knock-out mutants can often currently be obtained more easily with the Tnt1 insertion lines. Future developments in reverse genetics tools will certainly include the use of TALENs (transcription activator-like effector nucleases) or CRISPRs (clustered regularly interspaced short palindromic repeats) nucleases for genome modification (Gasiunas and Siksnys, 2013; Voytas, 2013).

Together with other mutated populations obtained by fast neutron, ethyl methane sulfonate (EMS), or gamma ray mutagenesis, the mutated populations used for reverse genetics have also been used for forward genetic screens, resulting in particular in the identification of numerous genes involved in symbiotic interactions with rhizobia or mycorrhizal fungi (Pislariu et al., 2012; see Chapter 63). Cloning of the mutated genes is easier in the case of Tnt1 mutants, although due to multiple insertions in a given line back-crosses with cosegregation analysis are often necessary. Moreover, the lengthy process of positional cloning in *M. truncatula* is made easier by the development of genomic resources: availability of a reference genomic sequence, resequencing of *M. truncatula* lines allowing unlimited genetic marker discovery, availability of microarrays for transcriptional cloning, and so on. The decreasing costs of sequencing open the way to direct genome sequencing of segregating populations to speed up the discovery of mutations causing a phenotype of interest.

78.6 TWO CASE STUDIES: GENE FAMILIES WITH REMARKABLE DIVERSIFICATION IN *Medicago*

Several gene families, particularly diversified in *M. truncatula*, are of interest. In particular, a group of genes encoding cystein-rich peptides (CRPs), also called defensin-like peptides, expanded in *M. truncatula* (Graham et al., 2004; Mergaert et al., 2003; see Chapter 67). These CRPs are in general short peptides with low sequence conservation. They have an *N*-terminal secretion signal in their nonmature form and are stabilized by disulfide bonds between intrapeptide cystein residues (Kondorosi et al., 2013). In plant genomes, this family seems to have expanded through ectopic and tandem duplication, followed by diversifying selection, leading to different subgroups, conserved or lineage specific. Although a minority have been functionally characterized, in plants members of this large family are known to play roles as antimicrobial agents or to be involved in various developmental processes (see Chapter 67). In the *M. truncatula* genome, about 800 CRP have been identified, most of them present in clusters containing 2–11 members often with pseudogenes, spread throughout the entire genome, compatible with their predicted mode of expansion (Young et al., 2011). Although a number of these genes are expressed in vegetative tissues or seeds (Tesfaye et al., 2013), a large majority have only been detected in nodules containing *S. meliloti* bacteroids. These were called NCRs (nodule-specific CRPs; see Chapter 67) and classified into 36 subgroups based on their sequence (Young et al., 2011). Transcriptomic analyses indicated that subsets of NCR genes have specific spatiotemporal expression profiles, some being expressed early in nodule cells just infected by rhizobia, others being more specific of cells containing fully differentiated bacteroids (Maunoury et al., 2010; Nallu et al., 2013). Conserved motifs that might correspond to *cis*-regulatory elements were identified in NCR promoter sequences, but the precise mechanisms of control of NCR gene expression remain unknown. Several pieces of evidence indicate that NCR peptides have a critical role in terminal bacteroid differentiation in *M. truncatula*: NCR peptides were found to be targeted to the plant cell secretory pathway and transported to bacteroids in symbiosomes (Van de Velde et al., 2010; see Chapter 67). A mutation in *M. truncatula* affecting NCR signal peptide cleavage and therefore NCR trafficking to bacteroids resulted in deficient bacteroid differentiation; treatment by NCR peptides of *S. meliloti* cultures induced cell elongation and an increase in DNA content similar to that seen in bacteroids; ectopic expression of NCRs in *L. japonicus*, a legume lacking NCRs, induced symptoms of terminal bacteroid differentiation (Kondorosi et al., 2013). However, targets of NCR peptides in bacteroids and their precise mode of action are unknown. In addition, it is not clear why such a large array of peptides is required for an efficient symbiosis in *M. truncatula*. Indeed no NCR encoding genes are detected in the genome sequences of several legumes that efficiently fix nitrogen in symbiotic association with rhizobia but do not form nodules with terminally differentiated bacteroids, such as *Glycine max* or *L. japonicus*. Although they have not been studied in great detail, NCR genes have been identified in legume genera related to *Medicago,* such as *Vicia*, *Pisum*, *Galega*, and *Trifolium*, which belong to the inverted-repeat lacking clade (IRLC) of legumes. Similar to *Medicago* species, these legumes form indeterminate nodules with terminally differentiated bacteroids showing amplification of bacterial genome as well as other modifications such as swelling. *Cicer* is another member of the IRLC clade forming indeterminate nodules but without swollen bacteroids (Oono et al., 2010). The recent sequencing of *C. arietinum* genome (Varshney et al., 2013) opens the way to characterization of CRPs in this species. Legume species hosting swollen bacteroids are found in other legume clades as well (Oono et al., 2010), it would be interesting to know whether NCRs play a role in these symbioses.

Another gene family that exhibits a great diversification in *M. truncatula* is that of the nucleotide-binding site leucine-rich repeats (NBS-LRRs), which confer resistance to a range of plant pests and pathogens through effector-triggered immunity. Nearly 800 NBS-LRR genes are found in the *M. truncatula* genome (Young et al., 2011), more than in the larger genomes of other legumes such as chickpea or soybean (187 and 506 NBS-LRR, respectively) and many other plants. More than 80% of these genes are found in clusters with an average of five genes/cluster distributed across chromosomes. Some very large clusters are found in regions showing a low conservation of synteny with other legume species and/or in regions with a high density of transposable elements (Young et al., 2011). Most of the NBS-LRRs belong to the TIR-NBS-LRR (toll/interleukin1 receptor) and CC-NBS-LRR (coiled-coil) groups that are found in similar proportions with a number of variants, particularly in the TIR class (Ameline-Torregrosa et al., 2008). Transcriptomic analysis following sequencing of mRNA in six different tissues showed that most of the identified NBS-LRRs genes are expressed in *M. truncatula* and allowed classification of NBS-LRR genes according to their expression pattern (Young et al., 2011). Interestingly, some of the genes were specifically induced in nodules, suggesting that members of this family could be involved in symbiotic interactions with rhizobia. Indeed in soybean, resistance genes were found to control host-specificity of the rhizobial–legume symbiosis (Yang et al., 2010). The presence of such a large number of NBS-LRR genes in *Medicago* raises one important question: how can the expression of these genes so important for biotic interactions be coordinated? It was recently found that NBS-LRR transcripts are targeted by networks of highly redundant small RNAs

(Fei et al., 2013, Zhai et al., 2011). NBS-LRRs are first targeted by 22 nt miRNAs in three unrelated families (miR2109, miR2118, miR1507) at several conserved motifs in the TIR or NBS domains. These miRNAs trigger the production of secondary siRNAs spaced in 21 nucleotide "phased" intervals, called phasiRNAs. These phasiRNAs, acting both in *cis* and *trans*, target related NBS-LRR transcripts, leading to regulation of their expression (Fei et al., 2013, Zhai et al., 2011). High levels of siRNAs matching more than 60% of NBS-LRR transcripts were detected, indicating that a majority of NBS-LRR genes can be regulated by this process. Production of secondary phasiRNAs amplifies the suppression effect of miRNAs, allowing regulation of most of the *Medicago* NBS-LRR genes. Similar processes were identified in other plant families like Solanaceae and to a lesser extent in Brassicaceae, but apparently not in Poaceae (Fei et al., 2013).

The functional relevance of the regulation of NBS-LRR gene expression by siRNAs remains to be precised. Several hypotheses have been proposed (Fei et al., 2013): suppression of NBS-LRR expression might be important for the establishment of beneficial biotic interactions, for example, with mycorrhizal fungi or rhizobia or it might play a role in plant defense against pathogens. Alternatively, such a regulation might allow steady-state regulation of NBS-LRR levels preventing accumulation of toxic level of NBS-LRR proteins, while allowing the preservation of NBS-LRR diversity necessary for response to pathogens. Further work is required to substantiate these hypotheses that are not mutually exclusive. It is also clear that the functional importance of this process varies among different plant families.

78.7 *M. truncatula* GENOME AND TRANSLATIONAL GENOMICS

One of the main incentives to develop genomic resources in *M. truncatula* was to facilitate analysis in phylogenetically related legume crops with less developed genetic and genomic resources, so-called orphan crops. Early on conservation of synteny between model and other legumes was used to facilitate positional gene cloning: once markers flanking the locus of interest were identified in the species of interest, new markers could be identified in the interval based on genomic information from the model legume, allowing fine mapping and identification of candidate genes (Endre et al., 2002; Limpens et al., 2003). On a larger scale, cross-species molecular markers were developed genome wide based on information from model legumes: PCR across introns using exon-specific primers allowed both amplification across species and discovery of polymorphisms (Choi et al., 2004a,b; Hiremath et al., 2012; Hougaard et al., 2008). The markers developed were used to construct genetic maps in different legume species, which confirmed the high overall conservation of synteny within the Papilionoideae subfamily. More recently, technical improvements and decrease in the costs of sequencing and genotyping allowed the development of genomic resources for orphan crops. In particular, transcriptome sequencing in various accessions of a given legume species led to the characterization of the gene space in these species as well as the discovery of abundant molecular markers (SNPs or SSRs (simple sequence repeats)) used to build high-density genetic maps (Bordat et al., 2011; Griffiths et al., 2013; Han et al., 2011; Hiremath et al., 2012; Sharpe et al., 2013). Comparison with genomes of model and other legume species shed light to the evolution of these genomes from a common ancestor, with a global conservation of synteny, and also many examples of rearrangements (translocations, inversions duplications, etc.). In addition, draft genome sequences of two legume crops, pigeon pea, and chickpea were published recently (Varshney et al., 2012, 2013), and that of common bean is on the way. Although these genome sequences were mostly assembled using next-generation sequencing data and genetic maps developed for these species, the *M. truncatula* genome assembly was used to tentatively orient 25% of the scaffolds of the chickpea genome (Varshney et al., 2013). Future assembly of the genome sequences of several important legume crops closely related to *M. truncatula* presents important challenges: large genome sizes due to abundant repeats (pea, lentil, faba bean), polyploidy, and heterozygosity (alfalfa, clover). These assemblies will certainly benefit from the *M. truncatula* genome assembly. Characterization of the gene space in these species through transcriptome or genome sequencing will also take advantage of the *M. truncatula* genome annotation to identify all members of various gene families, gene structure, splicing variants, and so on. In addition, comparative analysis of gene sequences in different legume genomes will identify orthologous groups as well as genes specific of some lineages or species, which might exhibit interesting functional diversification.

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Chapter 79

Leveraging Large-Scale Approaches to Dissect the Rhizobia–Legume Symbiosis

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79.1 INTRODUCTION

Legumes are of considerable agronomic and socioeconomic importance because of their ability to provide grains for human consumption, fodder for animals, as well as their use in the manufacturing of several industrial products (Graham and Vance, 2003). A hallmark trait of legumes is their availability to develop root or stem nodules and to fix nitrogen (N_2) in symbiosis with compatible rhizobia. This symbiotic interaction is critical for legume development in nitrogen-deficient soils. This interaction also helps to reduce soil and water contamination by reducing the need for synthetic fertilizers (Graham and Vance, 2003).

Legume–rhizobia interactions result in a new organ: the root nodule or stem nodule. Inside the nodule, the rhizobia fix atmospheric N_2 and transfer it to the plant in exchange for a carbon source. The development of this symbiotic process is generally characterized by a high level of host specificity and is coordinately regulated by the mutual exchange of diffusible signal molecules (Dénarié and Debellé, 1996; Long, 2001; see Chapters 50, 51). Flavonoids and isoflavonoids present in the root exudates act as specific inducers of the nodulation genes in the compatible rhizobia (Hartwig et al., 1990; Dénarié and Debellé, 1996). These bacterial nodulation genes are responsible for the production and secretion of bacterial lipochito-oligosaccharides (LCOs) known as Nod factors (NFs; Hartwig et al., 1990). NFs are essential for rhizobial infection and nodule development. Significant metabolic changes in both symbiont and host occur during the infection process and nodule development. For example, in the earliest stages of this interaction, the invading rhizobia trigger root hair deformation, depolarization of the membrane, cytoskeleton reorientation, calcium spiking, cortical cell division, the expression of several specific genes, and posttranslational modification (i.e., phosphorylation and ubiquitination) of a variety of proteins (Ehrhard et al., 1996; Felle at al., 1998; Cardenas et al., 2000; Stacey et al., 2006; Hervé et al., 2011; Popp and Ott, 2011; see Chapter 59).

This symbiotic interaction has been extensively studied on the model legumes *Medicago truncatula* (referred to as *Medicago*) and *Lotus japonicus* (referred to as *Lotus*). Based on forward and reverse genetic analyses, several genes involved in the initial stages associated with NF perception and transduction have been identified in these two model legumes. These studies indicate that NFs are perceived with high specificity by LysM receptor-like kinases (RLKs) localized in the plasma membrane (see Chapter 51). These kinases include Nod-factor perception (NFP), which is an essential component of a signaling receptor necessary for early response to NF and rhizobial infection (Ben Amor et al., 2003; Arrighi et al., 2006). Mutants in *NFP* are affected in calcium spiking, expression of early nodulin

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genes, and root hair deformation (Ben Amor et al., 2003). A leucine-rich repeats (LRRs)-receptor kinase, Does not Make Infections 2 (DMI2/NORK), is also localized on the plasma membrane and acts downstream of NFP (see Chapter 59). *Dmi2* mutants exhibit NF-induced root hair deformation, but are defective in NF-induced calcium spiking and rhizobial infection (Wais et al., 2000; Endre et al., 2002). Signals perceived at the plasma membrane are transduced downstream to activate signaling components residing at the nuclear level. DMI1, an ion channel, and MC8, a calcium pump, are localized on the nuclear envelope and control NF-induced calcium spiking (Ané et al., 2004; Peiter et al., 2007; Capoen et al., 2011). Calcium signatures are decoded by the calcium/calmodulin-dependent kinase (CCaMK) DMI3, which is localized inside the nucleus (Lévy et al., 2004; Messinese et al., 2007; see Chapter 54). DMI3 mutant plants are able to elicit calcium spiking and root hair deformation in response to NF, but cannot induce the expression of early nodulin genes or trigger cortical cell division (Wais et al., 2000; Lévy et al., 2004). Downstream of DMI3, two transcription regulators of the GRAS family, nodulation signaling pathway 1 and 2 (NSP1 and NSP2), control nodulin gene expression in an NF-dependent manner (Kaló et al., 2005; Smit et al., 2005; see Chapter 59). These two transcription factors (TFs) interact with each other and trigger the expression of Early Nodulin 11 (ENOD11) and Nodule Inception (NIN, Hirsch et al., 2009).

Although genetic approaches have led to the identification of different genes involved in the control of the legume–rhizobia interaction (some of them listed earlier), it is likely that additional "players" remain unidentified (see Chapter 63). For example, by using different large-scale analysis (i.e., transcriptomics, proteomics, and phosphoproteomics) in *Medicago*, *Lotus*, and *Glycine max* (soybean) treated with NF or interacting with rhizobia, several candidate genes have been identified (Serna-Sanz et al., 2011; Rose et al., 2012; Nguyen et al., 2012; see Chapter 78). Selecting the most promising candidates from the enormous amount of information generated by these "omic" approaches and then functionally characterizing them has become an imperative in this field. Here, we provide an overview of the information generated by transcriptomics and phosphoproteomics analyses of the legume–rhizobia interaction.

79.2 AVAILABILITY OF GENOME SEQUENCE: A MILESTONE FOR LARGE-SCALE ANALYSIS

New high-throughput sequencing technology and the accompanying bioinformatics tools have enabled the sequencing of several new plant genomes, including the genome of the two model legumes (*Medicago* and *Lotus*) and two of the most important legume crops (soybean and *Phaseolus vulgaris* (common bean)) (Schmutz et al., 2010; Young et al., 2011; http://www.phytozome.org/commonbean.php) (see also Chapter 78). Although the annotation and assembly of the genomes of these legumes are still in progress, about 90% of the expressed genes are represented in the current version (Schmutz et al., 2010; Young et al., 2011; http://www.phytozome.org/commonbean.php). For example, 47,845; 22,650; 73,320; and 316,338 genes have been annotated in the genomes of *Medicago*, *Lotus*, soybean, and common bean, respectively (Sato et al., 2008; Schmutz et al., 2010; Young et al., 2011). Even though these genomes are not completely assembled and annotated, the current genomic information has advanced the cloning and functional characterization of different genes and has allowed phylogenetic and syntenic analyses to be performed in these legumes (Li et al., 2011; see Chapter 78). Also, integration of the whole genome sequence with the genetic marker map that exist in some of these legumes has allowed cloning and identification of genes associated with particular quantitative trait loci (QTL, Grant et al., 2010, Joshi et al., 2012). Likewise, the genome sequence has allowed mutants to be identified and characterized more accurately. For example, after 3 years of the soybean genome sequence release, the generation of two mutant collections in soybean, one generated by fast-neutron mutagenesis and the other one by the insertion of the transposon *tnt1*, has been reported recently (Bolon, et al., 2011; Cui et al., 2013).

The availability of the genome sequences has also made it possible to accurately perform different large-scale analyses on these legumes. For example, to elucidate the molecular mechanisms that control the legume–rhizobia interaction, transcriptomic (both mRNA and smallRNA), proteomic, and phosphoproteomic analyses have been performed in *Medicago*, *Lotus*, and soybean plants treated with NF or in interaction with rhizobia (Libault et al., 2010; Boscari et al., 2012; Nguyen et al., 2012; Rose et al., 2012; see Chapter 78). These analyses have identified an enormous number of genes, proteins, and phosphorylation events that had not been identified by traditional genetic or biochemical analysis. Additionally, these -omic analyses have shown the overlap between different signaling pathways, for instance, the similarity between response to NF and the peptide flagellin (Serna-Sanz et al., 2011). These examples show how the availability of the whole genome sequence has positively impacted traditional genetic studies and also functional genomics studies in legumes.

79.3 TRANSCRIPTIONAL RESPONSES DURING THE LEGUME–RHIZOBIA INTERACTION

The initiation of the legume–rhizobia symbiosis triggers a variety of molecular and physiological changes in preparation for bacterial infection and nodule development. These changes are accompanied by different plant transcriptional events that lead to a successful interaction

(Oldroyd, 2013). Over the past two decades, different nodulin genes have been identified by forward and reverse genetic approaches (Sánchez et al., 1991; Pawlowski, 1997). One of these nodulin genes is the ENOD11, which is used as a marker gene to analyze the legume–rhizobia interactions (Journet et al., 2001). Likewise, two members of the GRAS family TF, NSP1 and NSP2, and the TF NIN are essential for both rhizobial infection and root nodule organogenesis (Kaló et al., 2005, Smit et al., 2005; Hirsch et al., 2009; see Chapter 59). In addition to the aforementioned genes, transcriptional analyses through DNA arrays or RNA sequencing (RNA-seq) approaches have demonstrated that a variety of genes show dynamic transcription during this symbiotic interaction (Libault et al., 2010; Boscari et al., 2012; Rose et al., 2012). For example, Mitra and coworkers (2004b) used a DNA microarray representing 9935 genes and identified 46 genes that were differentially regulated in *Medicago* plants exposed for 24 h to *Sinorhizobium melitoti* (*S. meliloti*; *Medicago* symbiont). Among these 46 differentially regulated genes, genes involved in ribosome biogenesis, plant-pathogen interaction, oxidative stress, and phosphorus transport were identified (Mitra et al., 2004b). Also, in this study, six Nod^- *M. truncatula* mutants (*nfp*, *dmi1*, *dmi2*, *dmi3*, *nsp1*, and *nsp2*) were used to demonstrate that these transcriptional responses are dependent on these six symbiotic genes (Mitra et al., 2004b). Likewise, these large-scale transcriptomic analyses have led to the discovery of different genes preferentially expressed in nodules or in roots following inoculation with rhizobia (Benedito et al., 2008; Libault et al., 2010; see Chapter 41).

Additionally, by combining transcriptome analysis on wild type (WT) and mutants of both symbionts (legume and rhizobia), genes involved in different stages of this symbiotic interaction have been identified (Mitra et al., 2004b). For example, in a study using a complementary DNA (cDNA) microarray representing 2366 genes, a two-stage reprogramming gene expression pattern was proposed to take place during the nodulation process in *Medicago* (Maunoury et al., 2010). However, a recent study with a 16,400 70-mer oligonucleotide microarray, in addition to the identification of 3400 differentially regulated genes, has led to the identification of four different stages of transcription reprogramming during the course of nodulation (Moreau et al., 2011). Based on this analysis, these four stages are (i) early signaling events and/or bacterial infection, (ii) plant cell differentiation dependent or (iii) independent on bacteroid differentiation, and (iv) nitrogen fixation (Moreau et al., 2011). Even within this classification, it is likely that each of these transcriptional stages could have complex subtranscriptional programming or transcriptional modules. Additionally, transcriptional studies in *Medicago*, *Lotus*, soybean, and common bean have helped to identify a variety of different transcriptional factors that play a role in each of the stages of this symbiosis (Libault et al., 2009).

It is likely that transcriptional reprogramming occurs immediately after NF perception. Since most of the transcriptional studies in the legume–rhizobia interaction (some of them mentioned earlier) have been focused on late responses, earlier transcriptional responses have not been described in detail. To address this, a recent study has reported the transcriptional changes that occur after 1 h of NF treatment in *Medicago* (Rose et al., 2012). In this study, RNA-seq (see Chapter 65) was used to analyze the transcriptional response to NF treatments in WT, *nfp*- and *dmi3 Medicago* mutant plants. One hundred and thirty-six genes were differentially regulated in response to NF treatment in the WT plants. The majority of these differentially regulated genes encoded for TFs, protein kinases, cell wall-modifying enzymes, defense-related proteins, transporters, and flavonoid biosynthesis proteins. Additionally, the ubiquitin E3 ligase PUB1, which interacts with the *Medicago* NF receptor LYK3 and negatively regulates nodulation, was upregulated after 1 h of NF treatment (Mbengue et al., 2010; Rose et al., 2012). Previously NFP was believed to be absolutely necessary for all NF-induced responses in *Medicago*. However, Rose and collaborators (2012) found that 31 genes were differentially regulated in *nfp* mutant plants after exposure to NF. This observation suggests the existence of other NFP-independent NF receptor complexes (see Chapter 51).

The molecular changes that lead to a successful legume–rhizobia interaction occur in specific root cell types. For example, the recognition of NF and the root colonization by rhizobia occurs in the root hairs (Stacey et al., 2006). The expression of genes that are essential for optimal nodule development and function is also cell type specific. Most of the transcriptional analyses on the legume–rhizobia interaction have used pooled tissue samples containing a mixture of different cell types. Because of the use of heterogeneous samples, it is likely that genes and gene-networks relevant for the symbiosis have not been identified yet. To overcome this problem, transcriptomic analyses have been performed in isolated root hairs after 24 and 48 h of rhizobia interaction in soybean plants (Libault et al., 2010; see Chapter 41). By using this single cell approach, different genes that preferentially express in the root hair with or without rhizobial interaction have been identified (Libault et al., 2010). Another approach that has been used to obtain cell type specific transcriptional data is laser capture microdissection (LCM). With this technique, the genes differentially regulated in zone II of *Medicago* nodules were identified (Damiani et al., 2012). Some of the differentially regulated genes in zone II are involved in glycolysis, mitochondrial electron transport/ATP (adenosine triphosphate) synthesis, and secondary metabolism (Damiani et al., 2012). Finally, additional efforts to integrate the transcriptional information, from either pooled tissues or single cell type studies, are necessary to better understand the transcriptional modifications that occur during this symbiosis. One of these efforts is the identification of gene regulatory modules or gene networks.

This analysis has been used to identify gene modules in soybean (Zhu et al., 2012). However, to date, there is no detailed report about the gene modules or gene networks that control the legume–rhizobia interaction.

79.4 MicroRNAs PARTICIPATE IN THE CONTROL OF THE LEGUME–RHIZOBIA INTERACTION

Like other physiological processes, the legume–rhizobia interaction is finely controlled at different levels. This interaction is controlled at the transcriptional level by different TFs, such as NIN, NSP1, NSP2, and HAP2 (Kaló et al., 2005, Smit et al., 2005; Hirsch et al., 2009). Recently, it has been demonstrated that the legume–rhizobia interaction is also regulated at the posttranscriptional level, primarily by microRNAs (miRNAs; Simon et al., 2009; see Chapter 78). miRNAs are 18–24 nucleotide, noncoding RNAs that control different developmental processes and nutrient homeostasis in both plants and animals (Mallory and Vaucheret, 2006). miRNAs play an important role in different stages of the legume–rhizobia interaction (Bazin et al., 2012). For example, miR166 and miR169 control the root and nodule development in *Medicago* (Combier et al., 2006; Boualem et al., 2008). Since the legume–rhizobia interaction is a multilayer process, it is likely that additional miRNAs are involved. To address this, different research groups have performed high-throughput sequencing analysis of small RNAs in different stages of the legume–rhizobia interaction (Subramanian et al., 2008; De Luis et al., 2012; Reynoso et al., 2013). These studies indicate that most of the miRNAs that are regulated during this symbiotic interaction belong to miRNA families conserved across different higher plant species (Subramanian et al., 2008). However, these analyses have led to the identification of novel miRNA families, some of which are present in legumes only (Subramanian et al., 2008). Some of these legume-specific miRNA families play a role in nodule development. For instance, the miRNAs such as miR482, miR1512, and miR1515 are positive regulators of nodule development in soybean (Li et al., 2010).

While some studies have investigated the significant transcriptional changes that occur during the first hour after NF perception (Rose et al., 2012), there are currently no reports about the miRNAs that are involved in the earliest stages of the legume–rhizobia interaction. However, in a recent study in rhizobia-inoculated *Lotus* roots, no significant changes in the abundance of miRNAs were found 3 h postinoculation (De Luis et al., 2012). Detection of plant-pathogen occurs in a manner similar to plant-symbiont recognition (Oldroyd, 2013). Plants can detect the presence of a pathogen through the detection of microbe-associated molecular patterns (MAMPs; e.g., flagellin and chitin) by pattern recognition receptors (PRRs), which are localized at the plant cell surface (Monaghan and Zipfel, 2012). Recently, it was demonstrated that PRR can be controlled by miRNAs (Li et al., 2012). Additionally, the abundance of different miRNAs (e.g., miR393) was significantly modified after 1 h of flag22 (a synthetic peptide of 22 amino acids from the conserved N-terminal of flagellin) treatment (Li et al., 2010). Due to the similarities between symbiont and pathogen detection, it is likely that some miRNAs play a role in the earliest stages of the legume–rhizobia interaction. Preliminary observations in the laboratory indicate that the abundance of miR2873c, miR2665, miR5207, and miR396c is significantly modified in *Medicago* roots after 1 h of NF treatment. Interestingly, putative targets of these miRNAs are E3 ligases. Two of these E3 ligases are SINA2 and SINA5, which play a role in nodule development in *Medicago* (Den Herder et al., 2008). This observation suggests that miRNAs can regulate some of the earliest responses of the legume–rhizobia interaction.

79.5 PROTEIN PHOSPHORYLATION: ANOTHER LEVEL OF REGULATION IN THE LEGUME–RHIZOBIA INTERACTION

During the legume–rhizobia interaction, a variety of proteins undergo posttranslational modifications. Some of these posttranslation modifications include ubiquitination and phosphorylation (Hervé et al., 2011; Popp and Ott, 2011). The initial steps in this symbiotic interaction involve specific receptor kinases and subsequent kinase cascades (Popp and Ott, 2011, Oldroyd, 2013). Protein phosphorylation is critical for the initiation of the legume–rhizobia interaction. The study of various plant mutants defective in nodulation led to the identification of key protein kinases essential for rhizobial infection and nodule formation. These include RLKs involved in the recognition of the NF signals, *MtNFP* (*GmNFR5* and *LtNFR5* in soybean and *Lotus*, respectively) and *MtLYK3* (*GmNFR1* and *LtNFR1*). Another important kinase is the nodulation receptor kinase (*NORK*, *MtDMI2*, and *SYMRK* in alfalfa, *Medicago*, and *Lotus*, respectively), a leucine-rich repeat receptor like kinase (LRR-RLK), first identified using a non-nodulating alfalfa mutant (Endre et al., 2002; see Chapter 59). The induction of calcium oscillations in the root hair cell is an important initial step in NF recognition and infection. These oscillations are likely detected and translated into cellular activity through the action of a CCaMK (Lévy et al., 2004; Mitra et al., 2004a; see Chapter 54). Transcriptional analysis in different stages of this symbiotic interaction indicates that the expression of different protein kinases is modified (Mitra et al., 2004b; Moreau et al., 2011; Rose et al., 2012). This observation suggests the participation of additional protein kinases as

well as the phosphorylation of additional proteins during the different stages of the legume–rhizobia interaction.

Phosphoproteome analysis allows for the survey of nearly all the phosphorylation events that occur in a particular physiological event. To identify the phosphorylation events that occur during the symbiotic interaction, phosphoproteome analyses have been conducted in *Medicago*, soybean, and *Lotus* plants (Grimsrud et al., 2010; Serna-Sanz et al., 2011; Nguyen et al., 2012; Rose et al., 2012; see Chapter 41). For example, 3457 unique phosphopeptides spanning 3404 nonredundant sites of *in vivo* phosphorylation on 829 proteins were identified in *Medicago* roots (Grimsrud et al., 2010). Interestingly, in this study, multiple sites of phosphorylation were identified on several key proteins involved in initiation of the legume–rhizobia interaction. Some of the proteins are Sickle (SKL), Interacting Protein of DMI3 (IPD3), and Nucleoporin 133 (NUP133). In an effort to characterize the phosphoproteomic changes that occur upon NF perception, a phosphoproteomic analysis was performed in *Medicago* roots treated with NF for 1 h (Rose et al., 2012). In this study, 98 differentially phosphorylated phosphoisoforms were identified. Interestingly, proteins involve in vesicle trafficking (e.g., Dynamin-related protein 2B (DRP2B)), ubiquitination (E3 ligases), translation (e.g., Eukaryotic translation initiation factor 5 (eiF5)), and proton pumping were differentially phosphorylated after 1 h of NF treatment. A similar phosphoproteomic analysis was conducted in soybean roots inoculated with rhizobia (Nguyen et al., 2012). In this study, the phosphorylation level of 240 proteins was modified after 30 and 60 min of rhizobial inoculation. These differentially phosphorylated proteins were classified into 24 functional categories. The largest functional groups were DNA–RNA related proteins (including TFs, DNA binding proteins, and chromatin-related proteins), signal transduction, protein trafficking, and cytoskeleton formation (Nguyen et al., 2012). Additionally, in both analysis in *Medicago* and soybean, a time course analysis revealed that most of the phosphorylation changes occur rapidly, that is, within 1 h after rhizobia inoculation or NF perception, whereas in later time points most the phosphorylation level decreases significantly (Nguyen et al., 2012; Rose et al., 2012). Likewise, a phosphoproteome analysis in *Lotus* roots treated with NF led to the identification of 13 differentially phosphorylated proteins (Serna-Sanz et al., 2011). Interestingly, the same 13 proteins were differentially phosphorylated upon flg22 treatment. This observation provides evidence about the overlapping in the symbiotic- and pathogenic phosphorylation pathways.

79.6 CONCLUSIONS

It is clear that large-scale analyses can provide valuable information and can help to dissect the legume–rhizobia interaction. However, how to integrate and interpret this information, it is one of the bigger challenges that researchers are facing. One possibility to deal with this is the inclusion of mutant plants and bacteria. But also, the integration of gene-network analysis, where data from any large-scale analysis (e.g., transcriptomics and phosproteomics) can be included, will help to integrate and obtain a biological sense from the large amount of data. Another challenge that we have to face is the selection of candidate genes for functional analysis. It is imperative to establish criteria that allow us select these candidate genes.

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Chapter 80

LegumeIP: An Integrative Platform for Comparative Genomics and Transcriptomics of Model Legumes

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80.1 INTRODUCTION

Legumes have been used as crops for harvesting oils, fiber, fuel, timber, medicinal, chemicals, and as horticultural varieties. As the third largest family of flowering plants, leguminous crops play important roles in raising productivity of farming in the temperate zone through building soil fertility and enhancing mulching quality. Legumes also have been well known for their ability to fix atmospheric nitrogen through symbiosis with nitrogen-fixing rhizobia, which is so highly specific that, with some exceptions, each rhizobial species interacts with only a specific group of legumes. Recently, the availability of genome sequences and expression data of multiple species such as *Medicago truncatula* (see Chapter 78), *Glycine max*, and *Lotus japonicus* opens the door to unravel the mystery of nitrogen fixing in legumes. Taking advantage of the data derived from multiple genome sequencing projects of the above-mentioned species, a large number of leguminous resources, such as legume Information (Gonzales et al., 2005), Soybase (Grant et al., 2010), MtGEA (Benedito et al., 2008), SoySeq (Severin et al., 2010), and LjGEA (Verdier et al., 2013), have been developed and comprehensive information for legume species can be queried and mined from these public data resources, which can be classified into two major types, sequence-based resources and transcriptome-based resources.

Soybase focuses on soybean breeding and research through integration information of genome sequences, gene expression, gene markers, and quantitative trait locus (QTLs). Other resources such as MtGEA, SoySeq, and LjGEA provide transcriptomics data from various stages of plant development in legume species. The development of these data resources provides a platform to understand

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the molecular regulatory mechanisms and cellular activity for crop improvement. While these data resources focus on the information from individual genes or species, the Legume Information System (LIS), a community portal hosts a large number of legume species and gene transcripts such as expressed sequence tags (ESTs), genetic markers, and literature and external links in multiple crop and model species (Gonzales et al., 2005).

Comparative genomics and transcriptomic approaches have entailed gene discovery and gene functional characterization, for example, Libault and Joshi et al. systematically reviewed many genes that were discovered and annotated through comparative analysis of sequences and expression data (Libault et al., 2009; see Chapter 41). The effectiveness of comparative genomics and transcriptomics of model legumes, coupled with comprehensive gene annotation and gene family catalogs along with phylogenetic analysis, have also been successfully demonstrated to be able to decipher unique complex legume biological processes, such as nodulation in response to rhizobial infection. For example, MtHAP2.1, MtERN, and LjNIN genes controlling nodule development were identified through analysis of collinear relationships and expression profiles (Combier et al., 2006; Middleton et al., 2007; Schauser et al., 1999; see Chapter 59).

In this chapter, we focus on the latest development of the comparative genomics platform, LegumeIP (Li et al., 2012), which is an integrative platform built on the integration of the genome-wide sequences and expression data sets for comparative genomics and transcriptomics of model legumes to facilitate the discovery of functional associated genes, study of gene functions, and genome evolution in legumes. We demonstrate the usefulness of our LegumeIP system through mining symbiosis-related gene across three legume species and nitrogen-fixation-related genes in *M. truncatula* from gene expression profiles and coexpression networks. The LegumeIP is publicly available at http://plantgrn.noble.org/LegumeIP.

80.2 OVERVIEW OF DATA REPOSITORIES

80.2.1 Data Integration from Original Database

Facilitated by traditional BAC (bacterial artificial chromosome)-by-BAC, whole genome shotgun, and second-generation sequencing technologies, the genomes of three legume species, *M. truncatula* (http://www.medicago.org/genome; see also Chapter 78), *L. japonicus* (http://www.kazusa.or.jp/lotus), and *G. max* (soybean) (http://www.phytozome.net/soybean) have recently been sequenced (Sato et al., 2008; Schmutz et al., 2010; Young et al., 2011). These sequencing projects provide invaluable resources for legume research. LegumeIP integrates the genome sequence information of these three legume species downloaded from their websites. Genome sequences of another model species, *Arabidopsis thaliana* were also downloaded and compiled from http://www.arabidopsis.org. LegumeIP also integrates the genome sequence information of another species, *Poplar trichocarpa*, to facilitate the functional comparison of nitrogen-fixation capability. Poplar genome sequences were downloaded from ftp://ftp.jgi-psf.org/pub/JGI_data/phytozome/v9.0/Ptrichocarpa/annotation/. In total, LegumeIP integrates 222,217 protein-coding gene sequences and 221,706 amino acid sequences. Furthermore, large-scale gene expression profiling for *Medicago truncatula* (http://mtgea.noble.org/) and *Glycine max* (http://digbio.missouri.edu/soybean_atlas/) have been performed to characterize tens of thousands genes in these legume genomes (Benedito et al., 2008; Hogslund et al., 2009; Libault et al., 2010). Large-scale microarray-based and RNA-Seq based gene expression data from *M. truncatula*, *L. japonicus*, and *G. max* were downloaded from http://mtgea.noble.org/, http://cgi-www.cs.au.dk/cgi-compbio/Niels/index.cgi, and http://digbio.missouri.edu/soybean_atlas/ respectively (Benedito et al., 2008; Combier et al., 2006; He et al., 2009; Hogslund et al., 2009; Libault et al., 2010). Additional expression data set were downloaded from public data repositories, such as the Array Express (Parkinson et al., 2011) and the GEO website (Edgar et al., 2002). Up to date, we have manually curated and integrated 104 microarray data sets for *M. truncatula*, 156 microarray data sets for *L. japonicus*, and 14 microarray data sets for soybean. Figure 80.1 shows the schematic workflow of genomics and transcriptomic data integration and analysis procedures for the construction of the LegumeIP.

80.2.2 Comprehensive Gene Annotation

The gene models were annotated by referring to a series of standard databases. The protein sequences were first queried against the UniProt databases (O'Donovan et al., 2002) using BLASTP with a cutoff e-value $\leq$1e$-$04. The top five meaningful query results were listed as valid annotations. Using the same BLASTP-based annotation protocol, the protein sequences were further annotated using the Gene Ontology (GO) database (Ashburner et al., 2000), the Kyoto Encyclopedia of Genes and Genomes Pathway (KEGG) database (Kanehisa et al., 2008), Transporter Classification Database (TCDB) (Saier et al., 2006), and PLANT Transcription Factor Database (PlantTFDB) (Guo et al., 2008). The conserved domains of protein sequences were identified by the InterProScan software using its default E-value cut-off thresholds (Hunter et al., 2009).

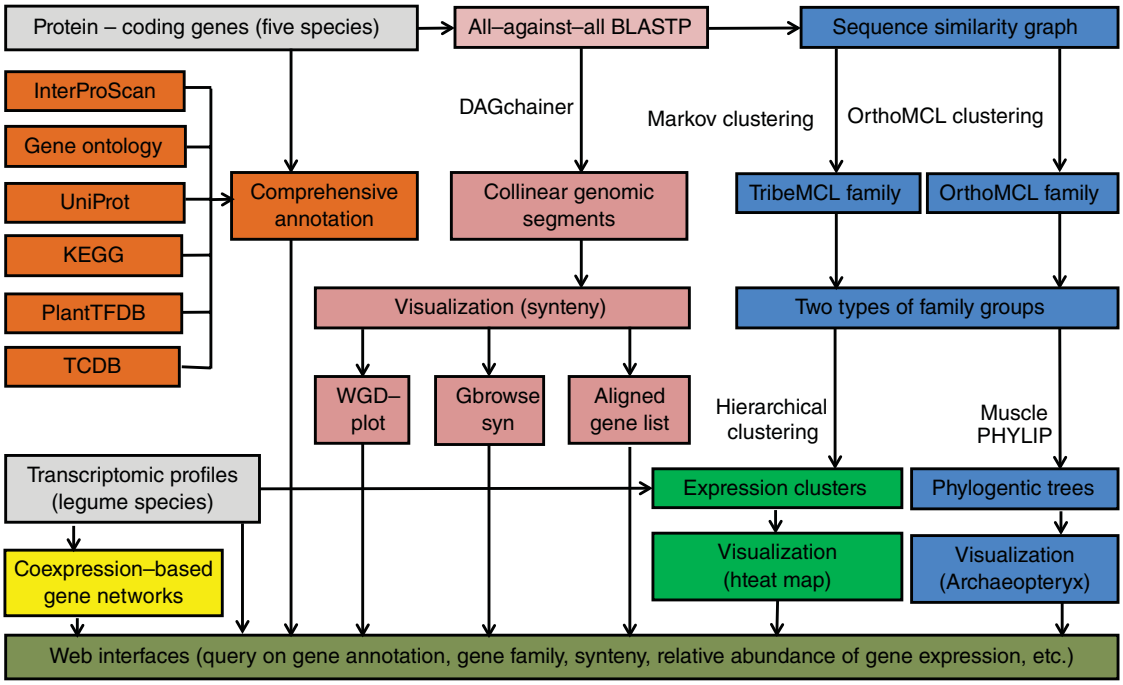

Figure 80.1 The schematic workflow on genomics and transcriptomics data integration and analysis for constructing the LegumeIP.

80.2.3 Systematic Synteny Identification

Aligning syntenic regions among legume species and non-legume species is an effective approach to identify patterns of evolution, conservation, and divergence of genome structures (see Chapter 78). The DAGchainer program (Eisen et al., 1998; Haas et al., 2004) was employed to identify syntenic regions among *M. truncatula*, *L. japonicus*, and *G. max* using *A. thaliana* as a reference outgroup. Briefly, BLASTN with e-value $\leq$ 1e−10 was applied to find intraspecies paralogous pairs and interspecies homologous pairs. DAGchainer with parameters, $Z = 12$, $D = 10$, $g = 1$, and $a = 5$, was then applied to identify orthologous pairs of interspecies collinear regions. Parameters with -s and -i in DAGchainer were used to find collinear homologous pairs in the same species. For all these identified homologous pairs in syntenic regions, we used the F3×4 model in the PAML4.0 package (Yang, 2007) to estimate the ratio of the number of nonsynonymous substitutions per nonsynonymous site (Ka) to the number of synonymous substitutions per synonymous site (Ks), that is, Ka/Ks.

80.2.4 Cross-Species Gene Family and Phylogenetic Analysis

Due to the frequent occurrence of tandem duplication or sequence diversity, orders of orthologous genes in collinear/syntenic regions may have been disrupted. To better understand gene function and genome evolution, two sizes of putative gene families were constructed in five species based on the protein-coding sequence similarity using two algorithms. First, the TribeMCL clustering algorithm (Enright et al., 2002) was employed to construct gene families of larger sizes. To circumvent the high false-positive rate generated by the nature of spurious BLAST hits within the TribeMCL algorithm, the OrthoMCL (Li et al., 2003), a low false-positive sequence clustering algorithm was adopted to construct gene families with smaller family size based on the same protein similarity graph. Briefly, first, all-by-all BLASTP was performed between coding nucleotide sequences in five species with parameter e-value $\leq$1e−10. Second, the TribeMCL with default parameter $I = 2.0$ was used to delineate corresponding gene families of larger family size based on the BLAST results. Third, the OrthoMCL was used to find more stringently defined homologous pairs, then the Markov cluster (MCL) method with default parameter $I = 2.0$ was used to construct gene families with small size but at lower false-positive prediction rate. In total, 95.70% of protein-coding genes (212,653 genes) were grouped into 12,166 gene families by the TribeMCL method, and 70.40% of all protein-coding genes were classified into 19,315 gene families by the OrthoMCL method.

To construct phylogenetic trees for gene families, multiple sequence alignment was firstly performed by the MUSCLE software (Edgar, 2004). Unrooted trees were then

created using PHYLIP software (including seqboot, proml, and consensus program) with 100 bootstrap replications.

80.2.5 Systematically Reconstruction of Genome-Wide Gene Coexpression Network and Identification of Functional Modules

Although large number of sequences could be annotated based on sequence similarity with public database such as GO, KEGG, TCDB, there still exists a large number of genes with unknown functions. Network-based gene-to-gene associated analysis provides a promising way to identify their functions. Genome-wide coexpression networks for the three legume species were therefore constructed using the DeGNServer (Li et al., 2013) and the large-scale collection of microarray data sets from public databases. These networks were further decomposed into multiple functional modules using the Markov clustering method (Enright et al., 2002). Based on the GO set enrichment analysis, the potential functions of those genes in the modules with unknown functions thus may be inferred based on other annotated genes in the same modules.

80.3 PLATFORM STRUCTURE AND WEB INTERFACE

LegumeIP was developed in Java and Groovy languages; it runs on a Linux-based RESIN J2EE web server architecture using MySQL as its database management system. The Circos software (Krzywinski et al., 2009) and Gbrowse (McKay et al., 2010) were adopted to visualize macrosynteny and microsynteny relationships, respectively. The Archaeopteryx (Han and Zmasek, 2009) was used to generate the interactive phylogenetic tree. The OpenFlashChart package (http://teethgrinder.co.uk/open-flash-chart/) was adopted to plot gene expression profiles. Gene cluster was visualized as a heatmap via HTML table coding.

80.3.1 Development of User-Friendly Web Interfaces for Data Access and Retrieval

LegumeIP provides a comprehensive set of web interfaces to search and explore genes, gene families, syntenic regions, and gene expression patterns. For example, through a simplified Keyword Gene Search interface, gene names, gene descriptions, specific biological functional categories as represented by GO terms, InterPro Domain names, KEGG compound names, a list of relevant gene IDs, gene family, and synteny regions of genes in multiple species can be searched. In the "Advanced Gene Search" page, more complex searching criteria can be created, such as combination of keywords with quantitative tissue-specific gene expression patterns. The retrieved results are usually listed in a summary page with links to batch download tools, the detailed gene page of comprehensive annotations, a plot of the transcriptomic profile if applicable, and sequence information along with "TribeGroup," "OrthoGroup," "Included gene in synteny" links to their corresponding synteny page and gene family page. The phylogenetic tree and heatmap-like table are included in the detailed gene family web page.

LegumeIP provides a simplified page to enable users to search or explore syntenic regions by either chromosomes or Contig IDs. The retrieved macrosyntenys, which include both interspecies and intraspecies syntenic regions, can be visualized as Circos maps and a summary table with links to web pages that contain more details of each gene using GBrowse syn, which is a Gbrowse-based synteny visualization tool.

LegumeIP also integrates BLAST search interfaces to allow users to search homologous genes or protein sequences based on sequence similarity.

80.4 DEMONSTRATIONS OF THE UTILITY OF LegumeIP

80.4.1 Mining SymRK Genes for Symbiosis Analysis in Legumes

Symbiosis with rhizobia in nodules is the source of nitrogen fixation of legumes. SymRK (symbiosis receptor-like kinase) genes play essential roles during establishment of endosymbioses in leguminous plants (Gherbi et al., 2008; Markmann et al., 2008). SymRK genes, which function as plant receptors in the symbiosome formation and endosymbiosis, are essential for the nod-signaling pathway in *L. japonicus.* An earlier study predicted more than seven SymRK genes in legume species (Markmann et al., 2008). Using the keyword "SymRK," we could successfully retrieve the seven genes from the LegumeIP. The results showed that these seven genes are classified in the same family, TribeMCL00867 group. Among seven genes, only four genes have corresponding probe sets presented on the Medicago GeneChip and all the four genes are highly expressed in nodules and roots. On the reconstructed phylogenetic tree, these expressed genes also located in the same clade (Fig. 80.2), which belongs to the same subgroup, OrthoMCL07722. The two gene families, TribeMCL00867 and OrthoMCL07722, have similar sequence features, and some of the member genes (Fig. 80.3) have similar gene expression patterns suggesting those genes coevolved with similar functions in legume root nodule symbiosis. Furthermore, the four genes are located in the corresponding syntenic regions (Fig. 80.4).

Figure 80.2 Phylogenetic tree of TribeMCL00867 family with the seven SyMRK genes indicated by the red box.

80.4.2 Mining Nodule-Specific Genes in *M. truncatula*

To mine nitrogen-fixation-related genes from *M. truncatula*, we used the "advance search" functions provided by our LegumeIP system to discover those genes that are highly expressed (e.g., the top 10% expressed genes) in nodules and roots, while low expressed (e.g., the lowest 30% expressed genes) in leaf and flower. Fourteen genes have been identified by LegumeIP; and nine genes are located in the third functional module/subnetwork (the list of genes in the subnetwork can be found at http://plantgrn.noble.org/LegumeIP/module.jsp?name=m2. It has been reported that in the ethylmethane sulfonate-induced snf1 mutant of *L. japonicus*, mutated *snf1* protein kinase (CCaMK, calcium-calmodulin-dependent protein kinase) is sufficient to turn fully differentiated root cortical cells into meristematic founder cells of root nodule primordia (Tirichine et al., 2006). Ca^{2+}/calmodulin genes are essential for CCaMK protein kinase to trigger *de novo* nodule formation in the absence of rhizobia or exogenous rhizobial signals (Tirichine et al., 2006; see Chapter 54). In *Medicago*, we also found a CCaMK gene (IMGA|Medtr8g043970.1), which is present in this module/subnetwork and also highly expressed in the nodule and root (Fig. 80.5), indicating the involvement of this module/subnetwork in nitrogen fixation. Furthermore, we found a nitrogen-fixation-related gene (IMGA|Medtr3g035970.2) interacting with the CCaMK gene. In addition, a nod-factor gene IMGA|Medtr8g0208401, whose orthologous gene in *Arabidopsis* is involved in the symbiotic signaling pathway (Bolle, 2004), was also present in this module. More importantly, for the nodule formation and nitrogen fixation, the gene function of *M. truncatula* CCaMK (MtCCaMK) is dependent on calmodulin-binding proteins (Routray et al., 2013). Through the autophosphorylation of S344 in the calmodulin-binding/autoinhibitory domain, the CCaMK

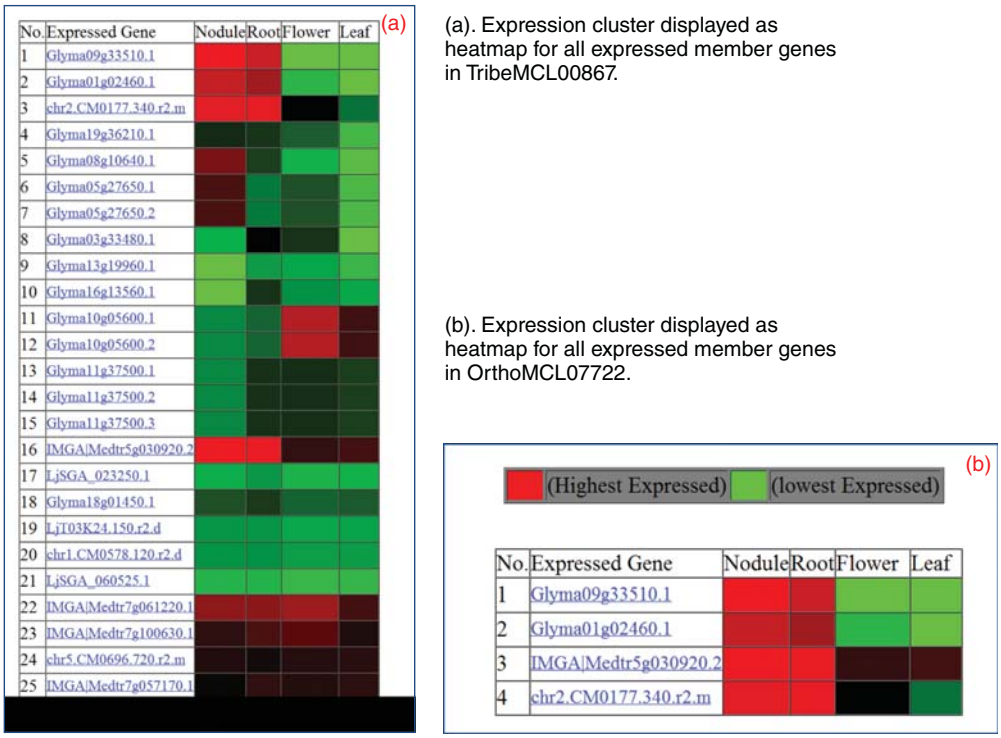

Figure 80.3 Gene expressions of two gene families, TribeMCL00867 and OrthoMCL07722, which include the SyMRK genes.

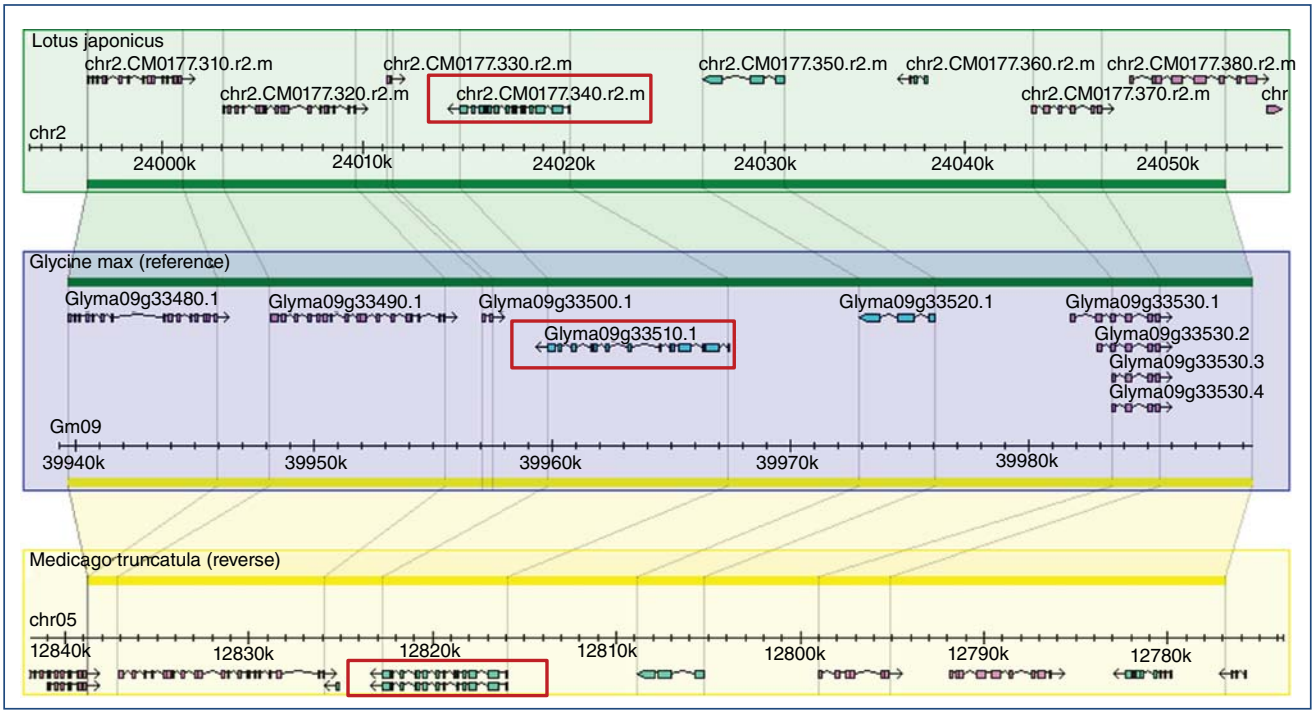

Figure 80.4 Orthologous genes of SyMRK gene family identified through collinear region analysis.

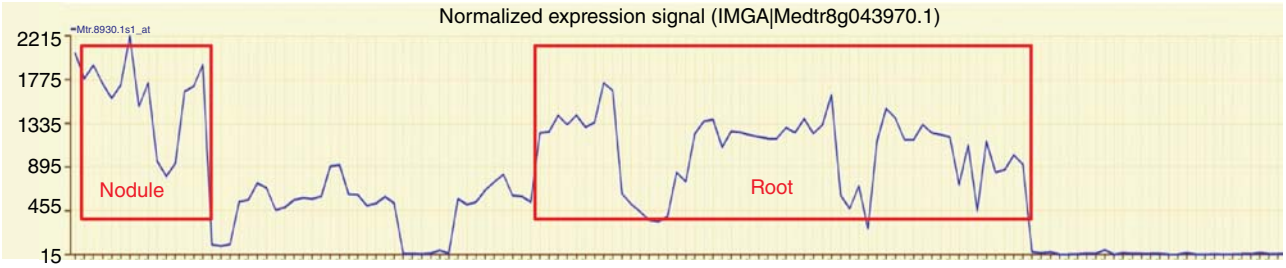

Figure 80.5 The tissue-specific expression of CCaMK gene (IMGA|Medtr8g043970.1) in *Medicago truncatula.*

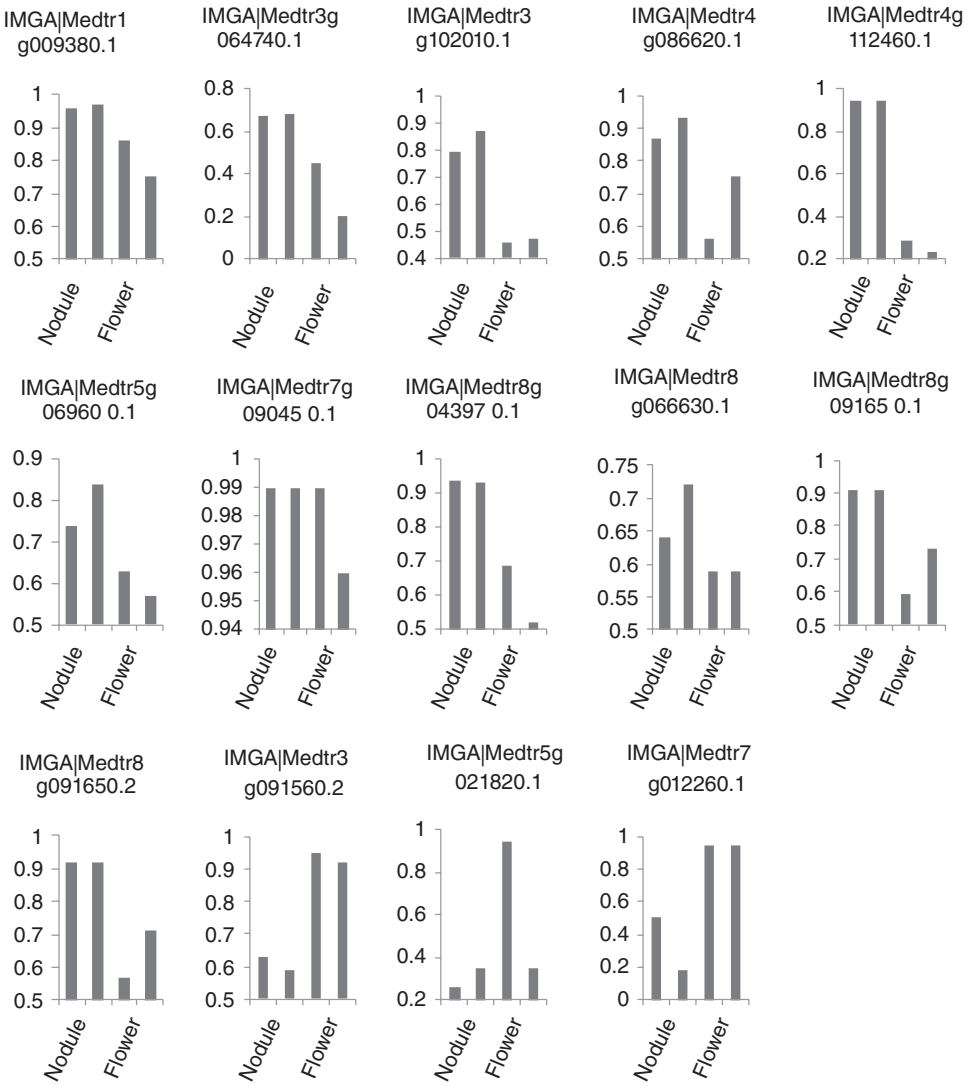

Figure 80.6 Most of the Ca^{2+}/calmodulin genes are preferably expressed in roots and nodules.

functionality was negatively affected during bacterial and fungal symbioses (Routray et al., 2013). In addition, the S337 residue is conserved across all angiosperm CCaMKs (Liao et al., 2012; Shimoda et al., 2012), and negative regulation of CCaMK protein by the S337 residue of CCaMK is essential for symbiotic infection in *L. japonicus*. In this module, more than 14 Ca^{2+}/calmodulin genes (IMGA|Medtr1g009380.1, IMGA|Medtr3g064740.1, IMGA|Medtr1g009380.1, IMGA|Medtr3g064740.1, IMGA |Medtr3g091560.2, IMGA|Medtr3g102010.1, IMGA|Medtr 4g086620.1, IMGA|Medtr4g112460.1, IMGA|Medtr5g021 820.1, IMGA|Medtr5g069600.1, IMGA|Medtr7g012260.1,

IMGA|Medtr7g090450.1, IMGA|Medtr8g043970.1, IMGA|Medtr8g066630.1, IMGA|Medtr8g091650.1, IMGA|Medtr8g091650.2) were found to be tightly connected with the gene CCaMK in the third subnetwork of *Medicago* coexpression network. In Figure 80.6, the sample genes that are highly expressed in the nodules are shown.

In the third module/subnetwork, we also found the presence of other genes that are involved in nitrogen fixation and assimilation. One of them is a nod-factor gene IMGA|Medtr8g089340.1, whose homolog in *Arabidopsis* encodes CAT8 (AT1G17120) that has been shown to participate in the symbiotic signaling pathway (Bolle, 2004). The CAT8 gene was identified as a glutamine transporter that functions at root tips with an activity independent of external pH (Su et al., 2004; Yang et al., 2010). Glutamine is one of the primary amino acids in nitrogen assimilation and often the most abundant amino acid in plant roots (Yang et al., 2010). IMGA|Medtr8g089340.1 also has the other homolog gene in *Arabidopsis* CAT1 (AT4G21120), which encodes a root-specific proton symporter to mediate high-affinity transport of basic amino acids already decades ago (Frommer et al., 1995). The other gene presented in the third module/subnetwork is IMGA|Medtr7g035060.1, whose homolog in *Arabidopsis*, NodGS (AT3G53180), belongs to TribeMCL03668 and its subgroup OrthoMCL02529. NodGS encodes a fusion gene with an N-terminal part sharing homology with nodulins. It is a nodulin/glutamine synthetase-like protein (glutamine synthetase is a key enzyme in nitrogen assimilation (Doskocilova et al., 2011)). In nitrogen/glutamate metabolism, the NodGS gene was found to affect the pool of endogenous glutamate/glutamine and also root morphogenesis in consequence (Doskocilova et al., 2011). All above-mentioned evidence suggests that the third module/subnetwork has cohesive functions related to nitrogen fixation through integrating the components of CCaMK, as well as signaling components.

Taken together, LegumeIP system comprises comprehensive sequence and information-enriched expression data and enables users to discover biological knowledge, such as genes/functional modules/subnetworks that are closely associated with nitrogen fixation.

80.5 CONCLUSIONS AND FUTURE PERSPECTIVES

LegumeIP hosts comprehensive genomics data related to nitrogen fixation from multiple species and the analytic tools that can be used to not only extract but also analyze information from these data, such as to retrieve comprehensive BLAST-/InterProScan-/GO-/KEGG-based gene annotations, calculate the relative abundance of gene expression, and perform gene family and macrosynteny analysis. The data and analysis tools are thoughtfully arranged and presented. Users are able to quickly search for the genes of their interests through friendly designed web interfaces. Meanwhile, the transcriptomics profiling, synteny, and phylogenetic analysis are powerful tools for gene function identification and annotation. For example, identification of genes involved the nodule formation process, one of essential biochemical reactions for the proper functioning of the biosphere. Furthermore, synteny and phylogenetic analyses provide information for scientists to better understand evolution of terrestrial plants, especially legumes. These functionalities and capabilities manifest the great potential of LegumeIP in studying fundamental questions of common interest in biology.

Aiming at a long-term empowering of the comparative genomics and transcriptomics of model legumes, we are committed to continuous improvement of the LegumeIP. In the near future, we will populate more sequences, microarray gene expression data, RNA-seq data from currently included and additional model and crop legume species into the LegumeIP database. Furthermore, we plan to integrate large-scale single-nucleotide polymorphism (SNPs) and genome-wide association studies (GWAS) data from various sources, for example, the *Medicago* Hapmap project (http://www.medicagohapmap.org/), which will deliver large-scale genomic information associated with 384 inbred lines spanning the range of *Medicago* diversity. These resources will further expand our knowledge related to SNPs, GWAS, and gene evolution, and better cover those unannotated genes without corresponding hits in the reference database, or without corresponding expression data due to lacking of present probe sets on the arrays.

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Chapter 81

Databases of Transcription Factors in Legumes

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81.1 INTRODUCTION

The availability of genome sequences of soybean (*Glycine max*) (http://www.phytozome.net/soybean), *Lotus japonicus* (http://www.kazusa.or.jp/lotus), and *Medicago truncatula* (http://www.medicago.org/genome; http://www.phytozome.net/medicago; see Chapter 78) has opened an opportunity for comparative genomic studies of transcriptional regulatory networks, which are controlled by sequence-specific DNA-binding transcription factors (TFs), in these legume species (Sato et al., 2008; Schmutz et al., 2010; Young et al., 2011). Among these three leguminous plants, soybean has been known as a very important crop worldwide, providing an abundant source of oil and protein for human consumption and animal feed (Manavalan et al., 2009; Tran and Mochida, 2010a; Thao and Tran, 2012; Ha et al., 2013). In addition, one of the prominent features of the legumes is their ability to fix atmospheric nitrogen through the symbiotic interaction with rhizobia, which allows them to grow under limited nitrogen sources (Sulieman and Tran, 2013; Sulieman et al., 2013a, b; see Chapter 99).

TFs that bind to DNA and either activate or repress gene transcription control diverse biological processes, such as development, growth, cell division, and responses, to environmental stimuli (Riechmann et al., 2000; Guilfoyle and Hagen, 2007; Tran et al., 2007; Tran and Mochida, 2010a;

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Jogaiah et al., 2013). Typically, the TFs contain a distinct type of DNA-binding domain (DBD) and a transcriptional regulation region (TRR). However, in some cases, TFs may also contain other domains, such as protein–protein interaction domains or transmembrane motifs that play important roles in the regulation of the TF actions (Guilfoyle and Hagen, 2007; Seo et al., 2008; Tran et al., 2009; Le et al., 2011; Puranik et al., 2012). The specific interactions between TFs and their specific binding sites, the so-called *cis*-regulatory sequences that are contained in the promoter regions of genes, affect spatial and temporal gene expression, thereby playing a central role in the regulation of various biological processes. The TFs and their *cis*-acting motifs together function as molecular switches for gene expression as well as terminal points of signal transduction in the regulatory networks.

With approximately 4–7% of the total genes in a plant encoding TFs (Udvardi et al., 2007; Perez-Rodriguez et al., 2010; Mochida et al., 2011; Zhang et al., 2011; Mochida et al., 2013), which in turn control the expression of the whole genome, it would be ideal to perform functional studies of the TFs at the genome-wide level to gain a whole picture about their regulatory functions (Riechmann et al., 2000; Tran and Mochida, 2010b, 2010c). The completion of genome-sequencing projects of many plants, such as *Arabidopsis thaliana* (Arabidopsis Genome Initiative, 2000), rice (Sakai et al., 2013), poplar (Tuskan et al., 2006), maize (Schnable et al., 2009), sorghum (Paterson et al., 2009), and legume species (Sato et al., 2008; Schmutz et al., 2010; Young et al., 2011), and the development of high-throughput -omics techniques (Ma et al., 2012; Jogaiah et al., 2013; Komatsu et al., 2013) have enabled scientists worldwide to identify TF repertoires in various plant species to provide a basic foundation for comparative and functional studies at a genome-wide level (Mochida et al., 2009, 2010, 2011, 2013; Yilmaz et al., 2009; Perez-Rodriguez et al., 2010; Zhang et al., 2011). In this chapter, we briefly summarize the databases describing TF repertoires from three model legumes: *L. japonicus*, *M. truncatula*, and soybean. These are either species-specific databases that house TF repertoire of a single-legume species or integrative databases that contain TF repertoires of many plant species including the leguminous species.

81.2 SPECIES-SPECIFIC DATABASES FOR LEGUME TFs

Recently, the genome sequences of soybean, *L. japonicus* and *M. truncatula*, have been made available to public (Sato et al., 2008; Schmutz et al., 2010; Young et al., 2011; see Chapter 78). Taking advantage of this, different research groups have initially compiled the TF repertoires from these species for large-scale functional studies of their TFs. Among the TF repertoires of these three leguminous plants, only the TF set of soybean was reported in soybean-specific databases in addition to integrative databases. These specific databases were made available to soybean scientists at concurrent time in 2009 by two independent research groups from Japan (SoybeanTFDB) (Mochida et al., 2009) and the United States (SoyDB) (Wang et al., 2010).

The SoybeanTFDB database (http://soybeantfdb.psc.riken.jp) hosted by the RIKEN Plant Science Center (currently known as RIKEN Center for Sustainable Resource Science) of Japan used the earliest annotation version of the soybean genome sequence (Glyma1.0 model) provided by Phytozome for the identification of the TF set of soybean. This database provides access to 4342 genetic loci encoding 5035 TF models that are classified into 61 families based on their DBDs (Mochida et al., 2009; Tran and Mochida, 2010b). This soybean TF set was compiled by using 51 HMMs (hidden Markov models) of Pfam and those of 11 HMMs, which were originally created using HMMbuild of the HMMER package (http://hmmer.janelia.org/), to search against the proteome data set of Glyma v1.0. The search results for each TF family were then analyzed to retrieve discovered regions as conserved DBDs and relevant annotation information. SoybeanTFDB basically allows researchers to browse predictions for all the predicted 5035 TF models and to receive classifications for their respective submitted nucleotide and protein sequences. Significant information such as functional motifs, domain alignments, promoter regions, genomic organization, and putative regulatory functions based on annotations of gene ontology (GO) inferred by comparative analysis with *Arabidopsis* is available for each soybean TF. Multiple alignments of amino acid sequences within TF families are also available for downloading and can be used for the construction of phylogenetic trees. In addition, clustering results showing amino acid similarity with different levels of amino acid identity (30%, 60%, and 90%), search functions for functional motif information of InterProScan, *cis*-motifs in promoter regions of TFs and GO annotations are also provided in the database. Furthermore, cross-references and links to other databases, such as *Arabidopsis* TAIR8, TIGR rice, UniProt, SoyBase, soybean FL-cDNA, as well as other TF databases, either species-specific, such as AtTFDB (Davuluri et al., 2003), DATF (Guo et al., 2005), RARTF (Iida et al., 2005), DRTF, or integrative TF databases, such as Grassius (Yilmaz et al., 2009), PlnTFDB (Perez-Rodriguez et al., 2010), are also supplied. This feature provides a unique option for comparative studies of TF repertoires among various plant species. Hyperlinks linking the soybean TF-encoding genes to the available FL-cDNA clones (Umezawa et al., 2008), Unigenes, and Affymetrix Gene ID (Grant et al., 2010; Le et al., 2012) are also provided in detailed page of each TF to ease functional genomics research. Additionally, it is worthy to mention an interesting feature of SoybeanTFDB, which

provided information on known *cis*-elements predicted in the promoter region of each soybean TF. This feature enables those who have interest to easily carry out functionality predictions for any TF of interest based on the search results of *cis*-motifs and GO annotations. Soybean TFDB was perhaps the first TF database that provides such helpful information.

The SoyDB database (http://casp.rnet.missouri.edu/soydb/) built by Wang et al. (2010) identified more TFs −5671 genes encoding putative TFs in soybean, than did the SoybeanTFDB, although the two databases used the same annotated Glyma v1.0 version. These TFs were classified into 64 families based on the HMMs used in the study. The main components provided by SoyDB are as follows: protein sequences, predicted tertiary structures, DNA-binding sites, domain predictions, homologous proteins from the protein data bank, protein family classifications, multiple sequence alignments, consensus DNA binding motifs, and web logo of each family. Users can use five search keys "Text search," "PSI-BLAST search," "Browse database," "Family prediction by HMM," and "FTP" to access the provided information. Compared with the SoybeanTFDB, SoyDB did not provide the information for putative *cis*-motifs located in promoter region of each TF-encoding gene. However, it has an interesting feature that provided prediction of tertiary structure for those TFs, which have homologous template proteins in the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) (Berman et al., 2000), using the MULTICOM (Cheng, 2008).

81.3 INTEGRATIVE DATABASES FOR LEGUME TFs

There are several integrative databases housing TF repertoires of legume plants (Charoensawan et al., 2010; Ma et al., 2012). In this chapter, we focus on two major plant integrative databases, namely LegumeTFDB and PlantTFDB, which house TF sets of legume species.

The LegumeTFDB (http://legumetfdb.psc.riken.jp) was built by our group to provide a platform for comparative genomics of the TFs within legumes and beyond (Mochida et al., 2010). To continue the success with SoybeanTFDB, upon the availability of the genomic sequences of *L. japonicus* and *M. truncatula*, we computationally analyzed the annotated proteome data sets of *L. japonicus* and *M. truncatula* to identify their TF repertoires using the same approach that was used to compile the TF repertoire of soybean. All the relevant information that was provided for the soybean TFs were also supplied for the TFs from *L. japonicus* and *M. truncatula* on LegumeTFDB. Users familiar with SoybeanTFDB's interface can easily use LegumeTFDB to access the information provided for the TF(s) or TF families of their interest as these two databases have a similar design. For soybean researchers, an updated feature for soybean TFs was also provided in LegumeTFDB in comparison with SoybeanTFDB. The expression data for a total of 2411 soybean TF models (highlighted by red-colored letters in the search results of TF search page of each soybean TF family), including expression patterns for 32 different organs and tissues and those in response to several biotic stresses, such as treatments with *Phytophthora sojae* or *Phakopsora pachyrhizi*, housed on Genevestigator (https://www.genevestigator.com/gv/index.jsp) (Hruz et al., 2008), are accessible on LegumeTFDB through hyperlinks. All the search functions available on SoybeanTFDB are also found on LegumeTFDB as tools for comparative genomic analyses of TFs within these three important legume species or among the legumes and nonlegume model plants, such as *Arabidopsis*, poplar and rice. The expression data together with information of *cis*-motif analyses, GO annotations, and sequence similarities inferred from comparative analyses of the legumes can facilitate the systematic functional predictions of identified TFs (Tran and Mochida, 2010c).

The current PlantTFDB v2.0 (http://planttfdb.cbi.edu.cn) houses TF repertoires of 49 plant species, out of which 29 species have completed genomes (Zhang et al., 2011). In addition to the three well-known legumes – soybean, *L. japonicus*, and *M. truncatula* – PlantTFDB v2.0 also contains TF sets of *Arachis hypogaea* although its full genomic sequence has not been yet available. PlantTFDB classified the identified TFs into 58 TF families. Apart from the general information, including GO, domain feature, ortholog groups, expression patterns, and cross-references to various databases and literature citations, this database provided a unique feature that shows the multiple sequence alignments and phylogenetic trees for each TF family.

81.4 CONCLUSIONS

With their user-friendly interface, the databases described here meet the broad demands of researchers who are performing research on legume TFs with the goal of gaining greater understanding of their regulatory roles in different signaling pathways that control different biological processes, including plant development, differentiation, and environmental responses. Extensive research on the TFs has provided fruitful results, leading to a better understanding of the functions of the TFs, their regulatory networks, as well as the development of various crop varieties with improved performance under adverse growing conditions. With the future improvement in the annotation of the legume genomes analyzed by these databases and the availability of updated HMM libraries or refinements of existing ones, information provided by these databases needs to be occasionally fine-tuned and updated to improve the TF prediction accuracy, which in turn provides a great assistance to researches who focus on research of legume TFs.

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Chapter 82

Functional Genomics of Symbiotic Nitrogen Fixation in Legumes with a Focus on Transcription Factors and Membrane Transporters

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82.1 INTRODUCTION

Legumes, the third largest angiosperm family, have been utilized as important pulse crops for several millennia (Doyle and Luckow, 2003). In symbiosis with soil bacteria called rhizobia, leguminous plants significantly contribute to the global nitrogen cycle. Every year, approximately 40 million tons of nitrogen enter agricultural ecosystems through symbiotic nitrogen fixation (SNF) (Peoples et al., 2009). These features make SNF central to sustainable agriculture.

Symbiotic association between fungi from the phylum *Glomeromycota* and the majority of terrestrial plant species (70–90%) is known as arbuscular mycorrhizal (AM) symbiosis (Parniske, 2000). AM symbiosis emerged 400–460 million years ago (Mya). The origin of SNF, on the other hand, is dated back to around 60 Mya (Sprent, 2008). Forward genetic studies using model legumes (*Medicago truncatula* and *Lotus japonicus*) have shown that SNF evolved in part by recruiting and modifying the genetic pathways established in the ancient AM symbiosis (Oldroyd, 2013; Popp and Ott, 2011 called CSSP, CSP or SYM; see Chapters 54, 55, 108, 110). In contrast to AM symbiosis, SNF involves the formation of a new organ called the "nodule" and a new organelle-like structure the "symbiosome" in which rhizobia are entirely enclosed by a host cell membrane. During AM symbiosis, neither a new organ is formed nor is there complete endocytosis of the symbiont. Nevertheless, in both cases the host needs to generate a membrane interface equipped for nutrient exchange (Parniske, 2000; Sprent and James, 2007; see Chapter 68). Transcriptomic studies indicate that thousands of genes are expressed during SNF. For AM symbiosis, however, this number is in the hundreds (Gaude et al., 2012; Gomez et al., 2009).

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This difference supports the notion that SNF, though originating from AM symbiosis, has evolved as a much more elaborate, if not sophisticated, system.

In this chapter, we focus on two functional groups of genes, namely (i) transcription factors (TFs) and transcriptional regulators (TRs) as major determinants of genetic reprogramming during nodule development; and (ii) transporters that may be involved in exchange of nutrients between plant and rhizobia.

82.2 CURRENT KNOWLEDGE ON THE TRANSCRIPTIONAL CONTROL OF SNF IN LEGUMES

Classically, TFs are proteins that bind to specific DNA sequences and control the transcription from DNA to mRNA. A TR, on the other hand, does not necessarily have a DNA-binding domain (DBD); it influences transcriptions by altering transcription rates. A TR could be a transcription coregulator, a histone-modifying enzyme or even a DNA methyltransferase. Several TFs and TRs have been directly implicated in SNF, based on studies mainly using *M. truncatula* and *L. japonicus* as model systems (Table 82.1; see also Chapter 81). At least nine TFs and TRs have been genetically connected with nodulation. Although a few more TFs have been described, their mode of action remains elusive (Table 82.1). Briefly, four TFs are required at the early stage of bacterial recognition. Among them are two GRAS domain-containing TFs: *nodulation signaling pathway 1* (*NSP1*) and *NSP2* (Kalo et al., 2005; Smit et al., 2005). Mutations in these plant genes result in an inability to support infection thread (IT) formation (Catoira et al., 2000). It has been shown that NSP1 has a DBD, but NSP2 does not. NSP1 and NSP2 form a heterodimer that binds to the promoters of nodulation-specific genes, such as the TFs *nodule inception* (*NIN*) and *ethylene response factor required for nodulation 1* (*ERN1*) (Hirsch et al., 2009; see Chapter 59).

ERN1 is crucial at the early stage of Nod-factor signaling. This TF belongs to the AP2-EREBP family. Mutation in *ERN1* blocks the initiation and development of ITs. The *MtERN1* mutant *bit1-1* forms small bumps 2 months after inoculation with *Sinorhizobium meliloti* (Middleton et al., 2007). Further studies indicated that *ERN1* has a paralog in *Medicago*, called *ERN2* (Andriankaja et al., 2007; Young et al., 2011). *ERN1* is predominantly expressed during SNF,

Table 82.1 List of transcription factors and regulators required for SNF

Gene Name	TF Type	Mutagenesis and (Phenotype)	Reference*
MtNSP1/LjNSP1	GRAS	EMS/*Tnt1*; EMS (Nod-)	(Heckmann et al., 2006; Smit et al., 2005)
MtNSP2/LjNSP2	GRAS	EMS/*Tnt1*; EMS (Nod-)	(Heckmann et al., 2006; Kalo et al., 2005)
MtNIN/LjNIN	NIN-like	FNB/*Tnt1*; T-DNA) (Nod-)	(Marsh et al., 2007; Schauser et al., 1999)
MtERN1	AP2-ERFBP	FNB; EMS; *Tnt1* (Nod-)	(Middleton et al., 2007)
MtERN2	AP2-ERFBP		(Andriankaja et al., 2007; Cerri et al., 2012)
MtERN3	AP2-EREBP		(Andriankaja et al., 2007)
LjERF1	AP2-EREBP	RNAi (Fix+/−)	(Asamizu et al., 2008)
MtEFD	AP2-EREBP	RNAi, FNB (Fix-)	(Vernie et al., 2008)
MtHAP2-1/LjNF-YA1	NF-YA	RNAi/RNAi (Nod+/− Fix-)	(Combier et al., 2006; Soyano et al., 2013)
LjNF-YB1	NF-YB		(Soyano et al., 2013)
PvNF-YC	NF-Y-TF	RNAi (Fix-)	(Zanetti et al., 2010)
LjSIP1	ARID	RNAi (Fix-)	(Wang et al., 2013)
MtRR1	Cytokinin response regulator	RNAi (no phenotype)	(Ariel et al., 2012)
MtRR9/MtRR11	Cytokinin response regulator	RNAi (reduction in nodule primordium)	(Op den Camp et al., 2011)
MtRR4	Cytokinin response regulator		(Ariel et al., 2012)
MtbHLH476	bHLH	RNAi and *Tnt1* (reduction in nodule number)	(Ariel et al., 2012)
MtbHLH1	bHLH	CRES-T	(Godiard et al., 2011)
MsZPT2-1	C_2H_2	Antisense RNA (Fix-)	(Frugier et al., 2000)
MtNAC969	NAC	RNAi (premature senescence)	(de Zelicourt et al., 2012)
GmCND	MYB	RNAi (reduction in nodule number by 40%)	(Libault et al., 2009)
LjBzf	Leucine zipper RING-finger	EMS (enhanced and early nodulation)	(Nishimura et al., 2002)
MsNMHC5	MADS Box		(Heard et al., 1997)
MsNGL9	MADS Box		(Zucchero et al., 2001)
MtWOX5	HOMEOBOX	Ectopic expression (no phenotype)	(Osipova et al., 2012)
MtNOOT	BTB/POZ	*Tnt1* (emergence of roots from nodule apex)	(Couzigou et al., 2012)

Notes: Mt, *Medicago truncatula*; Lj, *Lotus japonicus*; Pv, *Phaseolus vulgaris*; Ms, *Medicago sativa*; Gm, *Glycine max*. Genes underlined are also involved in AM symbiosis.

*Only the most relevant references are mentioned.

whereas *ERN2* is expressed mostly during AM colonization. *ERN2* can functionally replace *ERN1* when expressed under the control of the *ERN1* promoter (Cerri et al., 2012). Another AP2-EREBP family member, the *ethylene-response factor required for nodule differentiation* (*EFD*), has been directly implicated in nodulation. Mutation in *EFD* causes a Fix- phenotype. EFD directly activates cytokinin signaling, which governs nodule differentiation (Vernie et al., 2008).

NIN is a plant-specific TF. It contains an RWP-RK domain conserved from algae to higher plants. Mutations in NIN block bacteria at ITs (Marsh et al., 2007; Schauser et al., 1999). Upon binding to DNA, NIN controls the expression of pectate lyase, which is crucial for IT formation (Xie et al., 2012). Several NIN targets have been identified by chromatin immunoprecipitation, gel mobility shift assay (GMSA), and inducible activation of NIN expression in transgenic plants. Among these targets are two NF-Y family members: LjNF-YA1 (*MtHAP2.1*) and LjNF-YB1. *NIN* overexpression induces nodule primordium-like structures, indicating that NIN and its direct targets (LjNF-YA1 and LjNF-YB1) control cortical cell division (Soyano et al., 2013; see Chapter 59).

Reverse genetic studies have connected an NF-Y family of TFs (MtHAP2-1/NF-YA1) with SNF (Combier et al., 2006). NF-Y TFs are heterotrimeric transcription factors usually composed of NF-YA (HAP2/CBF-B), NF-YB (HAP3/CBF-A), and NF-YC (HAP5/CBF-C) subunits (Laloum et al., 2013). RNAi knockdown (KD) of *MtHAP2-1* in *Medicago* indicates that this TF controls the development of the nodule meristematic zone. Bimolecular fluorescence complementation analyses in *Nicotiana benthamiana* leaves show that LjNF-YA1 and LjNF-YB1 interact with each other. Furthermore, in *Phaseolus vulgaris*, SNF is impaired by RNAi KD of NF-YC1 (Zanetti et al., 2010). Altogether, these results indicate that a heterotrimeric NF-Y complex is required for nodule development.

Two *MtbHLH* TFs have also been implicated in nodule development. Repression of *MtbHLH1* activity through chimeric repressor silencing technology (CRES-T) associated this gene with vascular patterning and nutrient exchange during SNF (Godiard et al., 2011). Another bHLH-TF (bHLH476) has been identified as a direct target of cytokinin signaling. A *Tnt1* retrotransposon insertion in this gene leads to a reduction in nodule number (Ariel et al., 2012).

Nodular Root (*NOOT*) is a homeotic gene coding for a BTB/POZ-ankyrin repeat protein that is necessary for the maintenance of nodule identity. Mutation in *MtNOOT* converts nodules to roots at various time points during nodule growth and development (Couzigou et al., 2012) (see Chapter 49).

Although nine TFs have already been shown to be essential for SNF, this number is probably the "tip of the iceberg" and certainly cannot account for the known complexity of transcriptional regulation associated with nodule development. We took a systematic reverse genetic approach to identify novel TFs and TRs required during SNF (see later).

82.3 FUNCTIONAL STUDIES ON MEMBRANE TRANSPORTERS INVOLVED IN SNF

Transporters facilitate nutrient exchange between closely interacting organisms and are essential for the establishment and maintenance of endosymbioses. A comprehensive summary of transport and metabolic processes relevant to the legume–rhizobia association was presented in a recent review (Udvardi and Poole, 2013; see also Chapter 68). To date, 22 transporter genes from nitrogen-fixing nodules have been cloned and functionally characterized (Table 82.2). The majority of these genes were discovered in legume species including *M. truncatula*, *L. japonicus*, *Glycine max*, *P. vulgaris*, *Vicia faba*, and *Lupinus angustifolius*. AgDCAT1 is the only SNF-related transporter cloned from a nonlegume species, *Alnus glutinosa* (Jeong et al., 2004). The existence of 11 more transport proteins active in nodules has been deduced using biochemical methods. In the case of the symbiosome membrane (SM), transport activities mediated by unknown transporters have been documented for H^+, malate, succinate, other carboxylates, glucose, and five forms of chelated Fe^{3+}, NH_4^+, Ca^{2+}, and K^+ (Andreev et al., 1999, 2005; Herrada et al., 1989; Kaiser, 1998; LeVier et al., 1996; Robertson et al., 1978; Udvardi et al., 1991; see Chapter 68). Furthermore, protoplasts derived from uninfected nodule cells have been shown to import sucrose, glucose, and amino acids, such as leucine (Peiter and Schubert, 2003; Peiter et al., 2004). In total, over 46 diverse substrates have been tested for transmembrane movement in nitrogen-fixing nodules. Direct or indirect evidence for subcellular localization is available for eight nodule transporters. Among them, GmNod26 (see Chapter 69), N70-homologs from soybean and *Lotus*, GmDMT1, GmZIP1, and AgDCAT1 are localized in the SM (Jeong et al., 2004; Kaiser et al., 2003; Masalkar et al., 2010; Moreau et al., 1998; Vincill et al., 2005), whereas LjAMT2;1, GmUPS1-1, GmUPS1-2, and GmPT5 probably reside in the PM of uninfected cells, including cells of the vascular tissue (Collier and Tegeder, 2012; Qin et al., 2012; Simon-Rosin et al., 2003). Mutant analyses of symbiotic transporters with potential roles in SNF in legumes have been described for only eight transporters. The first such transporter, LjSST1, was identified through both insertional (T-DNA) and chemical mutagenesis (EMS), whereas mutants for LjSEN1 and MtLATD/NIP (see Chapter 60) were isolated from EMS populations (Bagchi et al., 2012; Hakoyama et al., 2012; Krusell et al., 2005). More recently, LjPT3, GmPT5, GmUPS1-1, GmUPS1-2, and LjMATE1 have been studied by RNAi (Collier and Tegeder, 2012; Maeda et al., 2006; Qin et al., 2012; Takanashi et al., 2013).

Table 82.2 List of characterized transport systems associated with SNF

Gene Name or Source*	TCDB Family or Transport Type	Subcellular Location; Substrate(s)	Reference
La	ATPase	SM; H^+	(Domigan et al., 1988)
Gm	ATPase	SM; H^+	(Udvardi and Day, 1989)
GmNod26	NIP, 1.A.8.12	SM; NH_3, H_2O, glycerol, formamide	(Masalkar et al., 2010)
Gm	Carrier	SM; malate, succinate, oxaloacetate, fumarate, malonate	(Udvardi et al., 1988)
Pv	Carrier	SM; succinate, glucose	(Herrada et al., 1989)
Gm	Unknown	SM; Fe^{3+}-citrate, Fe^{3+}-anthranilate, Fe^{3+}-malate, Fe^{3+}-pyruvate, and Fe^{3+}-salicylate	(Moreau et al., 1995)
Gm	Channel	SM; NH_4^+	(Tyerman et al., 1995)
PvBHA1	P-ATPase, 3.A.3.3	PM; H^+	(Campos et al., 1996)
LjN70 GmN70	MFS, 2.A.1	SM; NO_3^-, NO_2^-, Cl^-	(Vincill et al., 2005)
GmDMT1	Nramp, 2.A.55	SM; Fe^{2+}	(Kaiser et al., 2003)
LjSST1	SulP, 2.A.53	?; $SO4^{2-}$	(Krusell et al., 2005)
Vf	ATPase	SM; Ca^{2+}	(Andreev et al., 1999)
LjLIMP1	TIP, 1.A.8.10	?; water	(Guenther and Roberts, 2000)
LjLIMP2	NIP, 1.A.8.12	?; water glycerol	(Guenther and Roberts, 2000)
LjSEN1	VIT, 2.A.89	TP?; Fe?	(Hakoyama et al., 2012)
Lj	Channel	SM; NH_4^+, ?Ca^{2+}	(Roberts and Tyerman, 2002)
GmZIP1	ZIP, 2.A.5	SM; Zn^{2+}, Cd^{2+}, Cu^{2+}	(Moreau et al., 2002)
LjKUP	KUP, 2.A.72	PM?; K^+	(Desbrosses et al., 2004)
LjAMT2;1	Not categorized	PM; NH_4^+	(Simon-Rosin et al., 2003)
LjSUT4	MFS, 2.A.2	TP; sucrose, salicin, helicin, maltose, sucralose, and synthetic phenyl glucosides	(Reinders et al., 2008)
Vf	Carrier?	PM; sucrose, glucose, Leu, Asn, Glu, Asp, Gln	(Peiter and Schubert, 2003; Peiter et al., 2004)
PvUPS1	SMR, 2.A.7.1	?; allantoin, xanthine, uric acid	(Pelissier et al., 2004)
AgDCAT1	POT, 2.A.17	SM; malate, succinate, fumarate, oxaloacetate	(Jeong et al., 2004)
LjABCB1	MDR, 3.A.1.201	PM?; IAA	(Takanashi et al., 2012)
Vf	Channel	SM; K^+	(Andreev et al., 2005)
MtLATD/NIP	POT, 2.A.17	?; NO_3^-	(Yendrek et al., 2010)
LjPT3	PHT, 2.A.1.9	?; PO_4^{3-}	(Maeda et al., 2006)
GmUPS1-1 GmUPS1-2	NBUT, 2.A.7.19	PM; allantoin, allantoic acid	(Collier and Tegeder, 2012)
LjMATE1	MATE, 2.A.66.1	?; citrate	(Takanashi et al., 2013)
GmPT5	PHT, 2.A.1.9	PM; PO_4^{3-}	(Qin et al., 2012)

La, *Lupinus angustifolius*; Gm, *Glycine max*; Pv, *Phaseolus vulgaris*; Lj, *Lotus japonicus*; Vf, *Vicia faba*; Ag, *Alnus glutinosa*; Mt, *Medicago truncatula*.
TCDB, Transporter Classification Database (http://www.tcdb.org); SM, symbiosome membrane; PM, plasma membrane; TP, tonoplast.
*The transport systems are listed in the order of their first biochemical characterization or gene cloning; the most relevant study is referenced in each case.

In this chapter, we report on symbiotic transporters identified on the basis of two transcriptomic resources (MtGEA and nodule zone profiling) and targeted by *Tnt1* insertional mutagenesis.

82.4 ESSENTIAL RESOURCES FOR FUNCTIONAL ANALYSIS OF SYMBIOTIC GENES IN *Medicago*

Model legume species are essential genetic systems for the discovery and characterization of genes central to SNF. In order to enhance the value of *Medicago* as a legume model, several genetic tools have been developed (see Chapter 78). Among them, the *Medicago* gene expression atlas (MtGEA) contains the most comprehensive transcriptomic data for all major organs of this species including a nodule developmental time series (Benedito et al., 2008). More than 26,000 genes are expressed during SNF, and 30.2% of them are differentially expressed more than twofold compared with roots. The version 2 of MtGEA contained 64 experiments and data from 156 Affymetrix chips. The database was recently updated to version 3 and now consists of 256 experiments and 670 chips. Importantly, this new MtGEA version includes transcriptomic data from several nodulation-related

TF mutants such as *efd*, *hap2.1*, and *noot* (Niebel et al., and Ratet et al., unpublished data). Furthermore, a large tobacco retrotransposon (*Tnt1*)-insertion mutant population of *Medicago* has been developed at the Samuel Roberts Noble Foundation, USA (Tadege et al., 2008). A detailed review of this valuable resource is presented in Chapter 83.

82.5 STRATEGIES OF CANDIDATE GENE SELECTION FOR REVERSE GENETIC STUDIES

We took several alternative approaches to identify genes for reverse genetic studies. Preliminary lists of nodule specific TFs and TRs are already present in MtGEA, they have been refined by annotation based on the current genome release (IMGAG 3.5v4). We started with annotation (Young et al., 2011) and re-annotation of TFs and TRs in the *Medicago* genome. Comprehensive annotation and transcriptional analysis of *Medicago* transporters based on MtGEA uncovered 50 nodule-specific and 196 nodule-enhanced transporters with transcript levels greater than fivefold compared to roots (Benedito et al., 2010). This annotation work presents an excellent source of candidate transporter genes for reverse genetic studies. We generated a new spatially resolved transcriptomic data set for *Medicago* nodule development (Pislariu et al., unpublished data). This useful data set aids in "smart" selection of candidate TF and transporter genes for functional characterization.

82.6 RE-ANNOTATION OF NODULE-INDUCED TF AND TRs

TFs are major determinants of any developmental program. According to MtGEA, 532 putative TF genes are differentially expressed (more than twofold) during SNF. It is conceivable that the de-differentiation of root cortical cells during early stages of SNF depends on epigenetic changes in chromatin structure. Accordingly, chromatin-remodeling enzymes and other chromatin modifiers might play key roles as TRs in differentiation and development of the nodule. We have re-evaluated the annotation of the 532 nodule-specific TFs using the IMGAG 3.5v4 gene models and online annotation tool PlantTFcat (plant transcription factor and transcriptional regulator categorization and analysis tool). We identified 417 TFs and TRs that were induced more than twofold during SNF. Of these, 358 are TFs and 59 are homeotic genes and chromatin regulators (TRs) (Fig. 82.1). Only 18 of the 417 genes have been directly implicated in SNF by genetic studies so far (Table 82.1). Given a very low proportion of characterized genes in this category, it is clear that a large number of nodule-induced TFs are potentially important for SNF, and thus deserve functional analysis. Among the 358 TFs induced during SNF, the most represented TFs belong to the C_2H_2 family (13%), followed by AP2-EREBP (13%), MYB (10%), WD-40 (9%), NAM (8%), C_3H, bZIP, bHLH (5% each), NF-Y, WRKY (4%

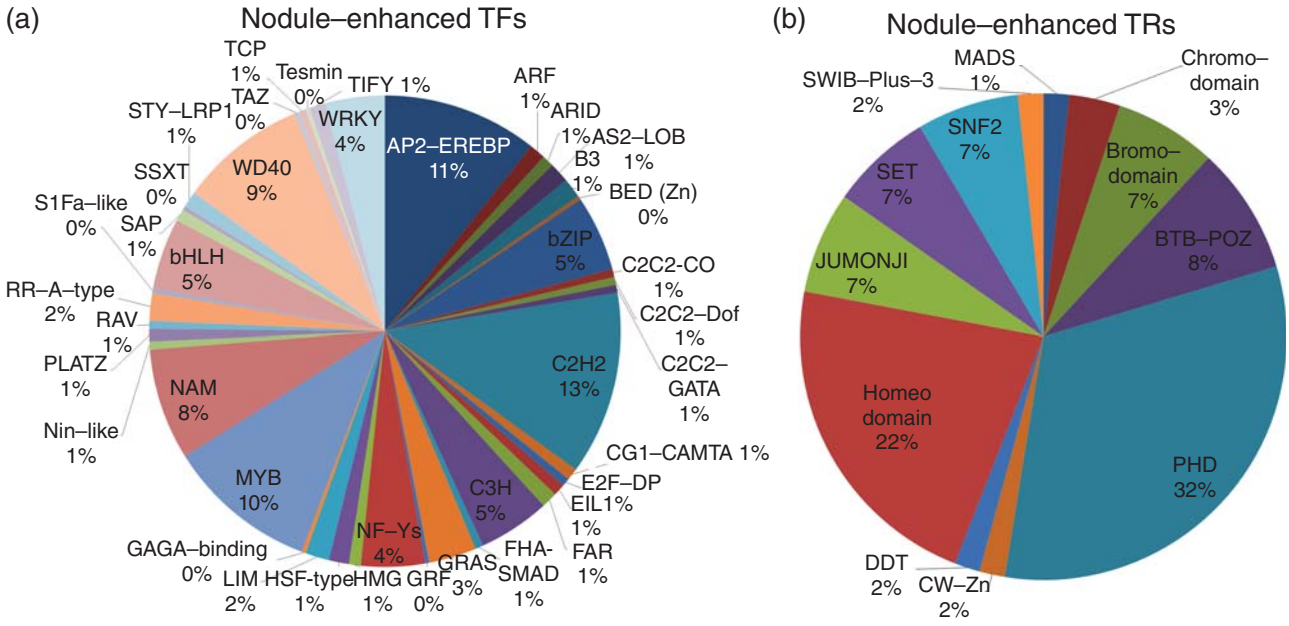


Figure 82.1 Re-annotation of *Medicago* nodule-enhanced transcription factors and regulators. Categorization of nodule-enhanced (≥twofold change of gene expression in nodules compared to roots) TFs and TRs in families is presented. Pie charts show the percentages relative to the total number of genes for TFs (a) and TRs (b) indicated by the number in the middle of each chart.

each), GRAS (3%), while the remaining 24% cover other TF families (Fig. 82.1a).

Among TRs and chromatin remodelers, most genes belong to the homeotic gene family represented by three subgroups as in Figure 82.1b: PHD, homeo domain, and BTB-POZ. In classical understanding, "homeotic gene" is a collective term for genes that control the pattern of body formation during early phases of organ development (Mukherjee et al., 2009). The most represented group of homeotic genes (32%) is the plant homeodomain (PHD)-containing family (Fig. 82.1b). PHD proteins usually reside in the nucleus and are involved in chromatin-mediated gene regulation. Most probably, they act by binding to trimethylated lysine residues of H3 histones (Sanchez and Zhou, 2011). The next major homeobox gene group (24%) is the homeobox domain family (Fig. 82.1b), which essentially encompasses a helix-turn-helix (HTH)-type DNA-binding structure that has been directly implicated in DNA binding, though the specificity of a single homeodomain protein is usually not enough to recognize only its preferred target genes, rather it requires additional protein–protein interactions for binding to promoter (Mukherjee et al., 2009). The third group of homeotic genes, the BTB-POZ domain-containing family (Fig. 82.1b), is also well represented (8%). The BTB-POZ domain is a protein–protein interaction module. It regulates gene expression through the local control of chromatin conformation. Other histone-binding protein families are the bromodomain-containing family (8%), Jumonji family (7%), chromodomain family (4%), DDT (2%), and CW-Zn (2%) (Fig. 82.1b).

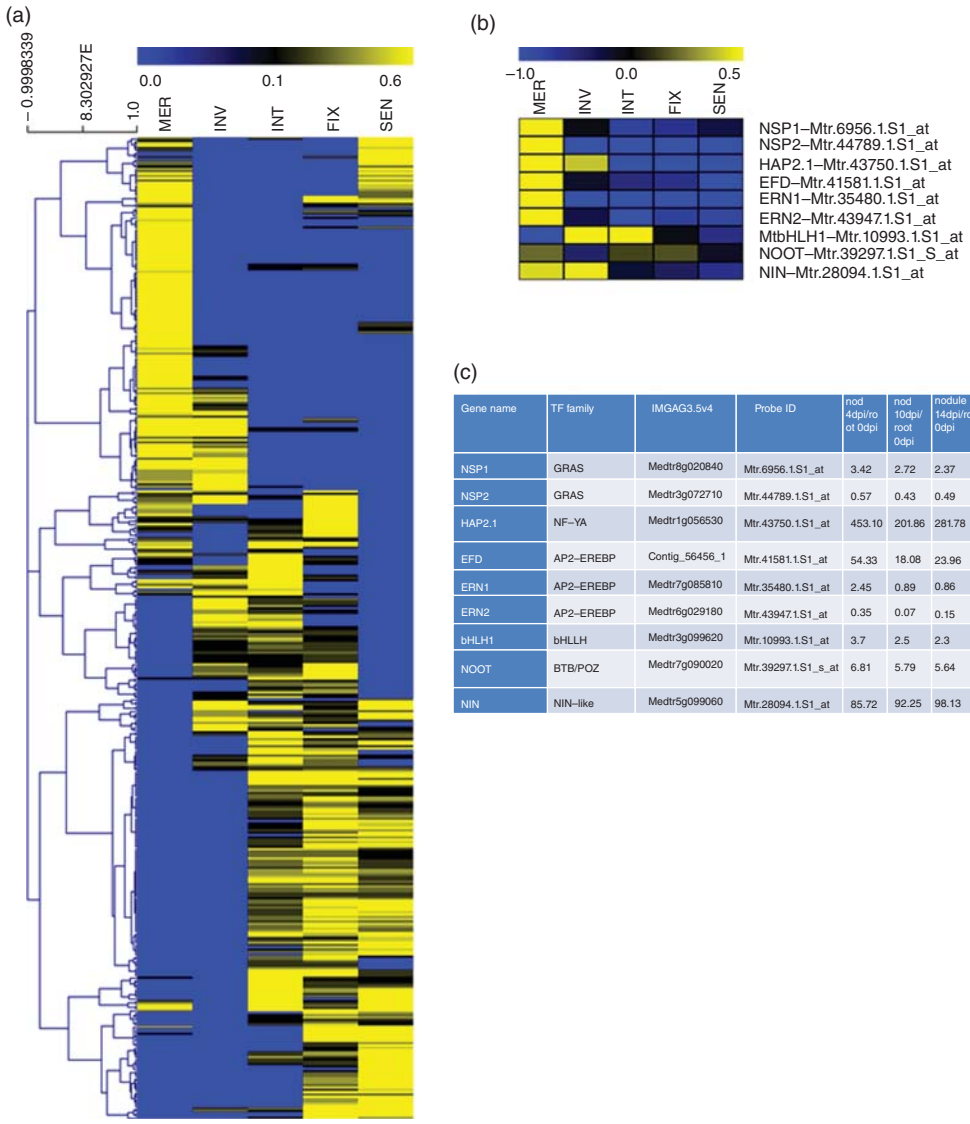
82.7 NODULE DEVELOPMENTAL SPATIOTEMPORAL GENE EXPRESSION PATTERN

Indeterminate nodules acquire cylindrical shape because of a meristem that remains active in the mature organ. A morphophysiological gradient can be observed in mature, nitrogen-fixing indeterminate nodules. *Medicago* forms indeterminate nodules that contain a persistent apical meristem (zone I), a bacterial invasion zone (zone II), a maturation interzones II–III, a nitrogen-fixing zone (zone III), and a senescence zone (zone IV) that is proximal to the root and only observed in older nodules (Vasse et al., 1990). Gaining insight into the spatial distribution of gene expression adds value to existing *Medicago* transcriptomic resources. To achieve this, we manually dissected nodules into five zones based on nodule morphology and the gradient of leghemoglobin color intensity. The Medicago Affymetrix Gene Chip was used to generate a global, nodule-specific, and spatially resolved transcriptome (Pislariu et al., unpublished data). The analysis of nodule zone-specific expression of TF and TR genes was conducted as described (Benedito et al., 2008). An overview of the zone-specific expression of TF and TR genes is shown in Figure 82.2a. The gradient of expression points toward the existence of functional groups and their potential linkage to zone-specific developmental and/or metabolic pathways. Before proceeding to selection of candidate genes, we verified that the spatial expression profiles of previously characterized nodulation genes matched expectation (data not shown). Nine TFs have been functionally characterized and shown to be critical for SNF. Among these, NSP1, NSP2, HAP2.1, EFD, ERN1, and ERN2 are induced very early during nodulation and have been shown to be critical for nodule initiation (see Chapter 59). Both MtGEA and our spatial data correlate well with published reports on these genes (Fig. 82.2b, c).

Although NIN is required for nodule initiation, its expression is not confined to the invasion zone; it is highly expressed throughout the nodule. Promoter-GUS analysis of NOOT and MtbHLH1 suggested transcript accumulation predominantly in the nodule vasculature (Couzigou et al., 2012; Godiard et al., 2011; see Chapter 49). Our results show a rather uniform expression of both genes along all zones, matching the distribution of vascular tissue in nodules.

82.8 SELECTION OF TRANSCRIPTION FACTORS AND TRANSPORTERS FOR REVERSE GENETIC SCREENING

MtGEA contains gene expression data from different time points of nodule development, beginning at 3 days postinoculation. Based on the temporal (Benedito et al., 2008) and spatial (Pislariu et al., unpublished data) expression patterns, we chose several TFs, TRs, and transporter genes for functional analysis. As a primary criterion, we chose genes that are expressed exclusively during nodule development, but we also did not eliminate those genes that have additional expression during AM formation. Furthermore, we paid attention to the genes specifically induced in the invasion and interzone, because the molecular events occurring in these two zones are crucial to nodule development in Medicago. A gradient of bacterial development from essentially "free-living" to the mature nitrogen-fixing form is seen within the invasion and interzones. Moreover, a few genes that satisfy the above criteria are also expressed during AM formation. These were given the highest priority. We isolated *Tnt1* mutants for 21 TFs and TRs (Sinharoy et al., unpublished data) (see also Chapter 83) and 20 transporter genes (Kryvoruchko et al., unpublished data). Lists of the genes that were chosen for reverse genetics, with preliminary phenotypes, are presented in Table 82.3 (TFs and TRs) and Table 82.4 (transporters). Reverse screening for the *Tnt1* mutants was performed as previously described (Cheng et al., 2011).



(c)

Gene name	TF family	IMGAG3.5v4	Probe ID	nod 4dpi/root 0dpi	nod 10dpi/root 0dpi	nodule 14dpi/root 0dpi
NSP1	GRAS	Medtr8g020840	Mtr.6956.1.S1_at	3.42	2.72	2.37
NSP2	GRAS	Medtr3g072710	Mtr.44789.1.S1_at	0.57	0.43	0.49
HAP2.1	NF–YA	Medtr1g056530	Mtr.43750.1.S1_at	453.10	201.86	281.78
EFD	AP2–EREBP	Contig_56456_1	Mtr.41581.1.S1_at	54.33	18.08	23.96
ERN1	AP2–EREBP	Medtr7g085810	Mtr.35480.1.S1_at	2.45	0.89	0.86
ERN2	AP2–EREBP	Medtr6g029180	Mtr.43947.1.S1_at	0.35	0.07	0.15
bHLH1	bHLLH	Medtr3g099620	Mtr.10993.1.S1_at	3.7	2.5	2.3
NOOT	BTB/POZ	Medtr7g090020	Mtr.39297.1.S1_s_at	6.81	5.79	5.64
NIN	NIN–like	Medtr5g099060	Mtr.28094.1.S1_at	85.72	92.25	98.13

Figure 82.2 Nodule zone-specific expression of transcription factor and regulator genes. (a) Hierarchical clustering of 417 nodule-enhanced transcription factors during nodule development. Clustering was performed using Pearson correlation. (b) Nodule zone-specific expression of nine TFs that have been functionally characterized and implicated in nodule development. For each probe set, relative expression was converted to the log2 value of the ratio representing the zone-specific expression over the average expression along all five zones. Data analysis was conducted on zone-specific transcriptomes generated in three biological replicates. (c) Induction in gene expression for nine TFs according to MtGEA temporal data at three time points with respect to 0 dpi. MER, meristem; INV, invasion zone; INT, interzones II and III; FIX, nitrogen-fixation zone; SEN, senescence zone.

82.9 ZINC-FINGER PROTEINS IN NODULE DEVELOPMENT

Zinc-finger proteins (ZFP) comprise the largest TF family in animals. They code for almost half of all annotated TFs in the human genome (Zhang et al., 2012). The classical Cys_2-His_2 (C_2H_2) zinc-finger domain was first identified in the *Xenopus* transcription factor IIIA (Hanas et al., 1983). Plant TFIIIA proteins usually contain one to four zinc fingers (Kubo et al., 1998). The phylogeny of nodule-enhanced proteins from this family is shown in Figure 82.3a. The most striking difference between plant and animal ZFPs is the presence of a highly conserved unique amino acid sequence (QALGGH) in the C_2H_2 DBD of the plant proteins (Takatsuji, 1999). Among the 19 nodule-enhanced C_2H_2-ZFPs, eight contain this consensus sequence. Half of these have two C_2H_2-DBDs while the other half have only one C_2H_2-DBD. The mouse Zif268 TF crystal structure suggests that the α-helix of zinc-finger domain interacts with the major groove of DNA. The zinc finger interacts

Table 82.3 Transcription factors that have been targeted for reverse genetics

IMGAG 3.5v4	TF Family	Putative Phenotype*	Line Information†
Medtr4g086190.1	AP2-EREBP	Fix+/−	Available
Medtr3g056160.1	C_3H-zinc finger	No obvious phenotype	Available
Medtr3g070880.1	C_3H-zinc finger	Fix+/−	Available
Medtr1g075430.1	C_2H_2-type	No obvious phenotype	Available
Medtr4g059870.1	C_2H_2 TF	Fix+/−	Available
contig_26399_1.1	C_2H_2 TF(RSD)	Fix−	Available
Medtr1g018420.1	C_2H_2 TF		No line
contig_55524_1.1	C_2H_2 TF	Fix−	Available
Medtr4g053250.1	bZIP		No line
contig_165403_1.1	bZIP	Fix+/−	Available
Medtr5g060940.1	bZIP		No line
Medtr3g099620.1	bHLH1‡	Vascular defect	Available
Medtr1g019240.1	bHLH		No line
contig_78503_1	NAM	Fix+/−	Available
Medtr5g089870.1	Homeobox	Fix−	Available
contig_17076_1.1	STY	No obvious phenotype	Available
Medtr3g014660.1	STY	No obvious phenotype	Available
Medtr5g021130.1	STY	No obvious phenotype	Available
Medtr7g009730.1	WRKY	Nod−	Available

*In many cases phenotypes are not yet confirmed by cosegregation analysis.

†Line information is provided based on the reverse screening results.

‡(Godiard et al., 2011).

Table 82.4 Examples of transporter genes targeted by reverse genetic screening

IMGAG 3.5v4	TCDB Family	Putative Phenotype*
Medtr4g108170.1	ABCA, 3.A.1.211	Fix−
Medtr8g036660.1	MATE, 2.A.66.1	Strong Fix−
Medtr4g077930.1	ABCB, 3.A.1.201	Fix−
contig_53079_1.1	P-DME, 2.A.7.4	No obvious phenotype
Medtr1g116930.1	POT, 2.A.17	Strong Fix−
Medtr8g103250.1	POT, 2.A.17	No obvious phenotype
Medtr6g086170.1	SulP, 2.A.53	No obvious phenotype
contig_64507_1.1	SWEET, 9.A.58	Mild Fix−?
Medtr3g098930.1	SWEET, 9.A.58	No obvious phenotype

*In many cases, phenotypes are not confirmed by cosegregation analysis.

with three successive DNA bases. This is controlled via hydrogen-bond interactions with amino acid residues in positions −1, 3, and 6 on the α-helix (Pavletich and Pabo, 1991). These three positions are usually hypervariable among different ZFPs, which confers variable specificities to the target. In the case of QALGGH-type ZFPs, two of the three hypervariable positions are present inside the QALGGH motif (underlined).

Among the QALGGH-type TFs, MsZPT2-1 (Krüppel-like Cys_2/His_2 ZFP) from alfalfa has been implicated in SNF (Frugier et al., 2000). Antisense *Mszpt2-1* plants were affected in the formation of SNF nodules. In these plants, initial root infection proceeded normally, whereas formation of the nitrogen-fixing zone was severely affected. Via a BLASTp search of the *Medicago* genome, we identified the *M. truncatula* ortholog of *MsZPT2-1* Contig_55524_1.1, whose protein is 92.8% identical to MsZPT2-1 (Fig. 82.3a, c). We also found three other nodule-induced TFs very closely related to MsZPT2-1, namely Medtr1g018420.1, Medtr2g096540.1, and Medtr7g100100.1. They belong to the same clade in the phylogenetic tree (Fig. 82.3a). Amino acid alignment indicates that these four proteins have two QALGGH-type zinc-finger domains (Fig. 82.3c). The three DNA interaction residues are identical to Medtr1g018420.1 and MtZPT2-1 (i.e., contig_55524_1.1). Other domains such as the NLS and the protein–protein interaction motif are highly conserved between Medtr1g018420.1 and MtZPT2-1 (Fig. 82.3c). The expression patterns of Medtr1g018420.1,

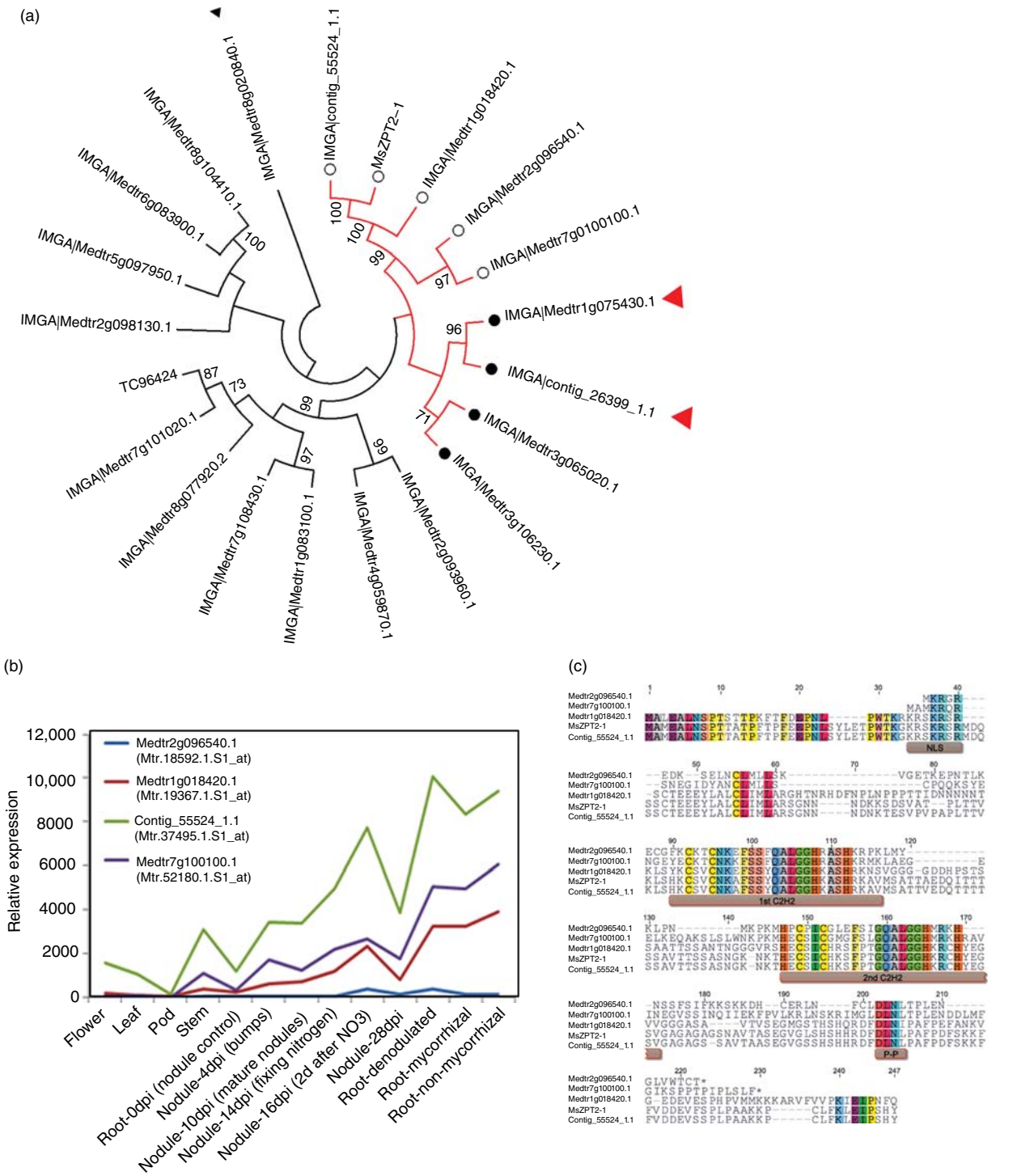


Figure 82.3 Nodule-induced C_2H_2 transcription factors. (a) Phylogenetic tree of nodule-enhanced C_2H_2 TFs from *Medicago truncatula*. Protein sequences from *Medicago* IMGAG 3.5v4 were used for the phylogenetic analysis. Medtr8g020840.1 (NSP1) served as a reference to root the tree, which was generated with MEGAv.5.1 (Tamura et al., 2011). TFs with QALGGH motif in the DNA-binding domain are highlighted with red in the tree. Zinc-finger proteins with two (unfilled circle) or one (filled circle) zinc fingers are marked. Proteins with EAR-type repressor motif are indicated by red arrowheads, and the outgroup is indicated by black arrowhead. The *Medicago sativa* ZPT2-1 protein (Frugier et al., 2000) was included in the analysis. (b) Expression profiles of two C_2H_2 zinc-finger domain-containing proteins according to MtGEA. (c) Alignment of two C_2H_2 zinc-finger domain-containing proteins. Protein alignment was carried out using MUSCLE (Multiple Sequence Comparison by Log Expectation) in the Geneious software suite (Biomatters). Two zinc fingers, NLS and protein–protein interaction domain (P–P) are highlighted.

Medtr7g100100.1, and contig_55524_1.1 are quite similar (Fig. 82.3b), indicating that they may play redundant roles during SNF. The antisense construct reported in the *MsZPT2-1* paper was generated against a 598-bp region between nucleotides 15–612 (Frugier et al., 2000). The nucleotide sequence of this region is 62.5% identical between *MtZPT2-1* (contig_55524_1.1) and *Medtr1g018420.1* (data not shown). Thus, the *Mszpt2-1* antisense construct might have targeted two or more homologs in alfalfa. Now, with the availability of the *Tnt1* mutant collection, it will be interesting to approach functional studies of MtZPT2-1 and Medtr1g018420.1 separately and in combination.

The second group of nodule-enhanced QALGGH-motif C_2H_2 TFs encompasses four single-finger classical ZFPs (Fig. 82.3a). Among these, two show nodule-specific expression. They have an *N*-terminal DBD and a *C*-terminal hexapeptide (DLELRL) called the EAR motif. This motif has been shown to confer transcriptional repression upon DNA-binding proteins (Hiratsu et al., 2003; Ikeda and Ohme-Takagi, 2009). Several proteins from this family have been functionally characterized and shown to play important roles in plant development (Kagale et al., 2010), but none of the EAR domain-containing proteins has been implicated in SNF until now. Of the two EAR domain-containing C_2H_2 TFs, contig_26399_1.1 is expressed 170-fold higher during SNF than in noninoculated roots, according to MtGEA. Apart from contig_26399_1.1, Medtr1g075430.1 belongs to the same EAR domain-containing C_2H_2 TF clade in the phylogenetic analysis (Fig. 82.3a). However, their sequences are quite distinct, which suggests that they may play different regulatory roles. We have shown that contig_26399_1.1 (*MtRSD*) plays a major role in the regulation of symbiosome development (Sinharoy et al., 2013, Table 82.3).

82.10 SNF-ASSOCIATED TRANSPORTERS TARGETED BY *Tnt1* INSERTIONAL MUTAGENESIS

We have selected 20 candidate transporters and isolated 52 *Tnt1* mutant lines, 41 of which carry transposons in exons of the target genes (see Table 82.4 for examples). Nearly all of the selected transporters are strictly nodule specific. Among these are mutants in two *Medicago* homologs of *GmNod26*. We also have *Tnt1* mutants for a *Medicago* homolog of *LjSST1*, a sulfate transporter required for effective SNF in indeterminate nodules. Preliminary analysis of the mutant phenotype (Fix+) suggests that in *Medicago*, the function of this transporter is probably redundant with that of another similar transporter(s). Three nodule-specific candidates for malate transport have also been tagged in the *Tnt1* population: two from the POT family and one from TDT family. Preliminary characterization of their phenotypes suggests that at least one of them, *MtPOT1*, is indispensable for SNF. Mutants in two nodule-specific SWEET transporter genes, *MtSWEET11* and *MtSWEET15*, are among the most interesting targets in our collection. SWEET transporters have recently been shown to be upregulated by bacterial and fungal pathogens and are thought to provide them with monosaccharides or disaccharides (Chen et al., 2010). These two genes are not expressed in AM, whereas a member of the SWEET family with expression in nodules and in AM roots has been identified recently (Harrison et al., personal communication). Given the existence of at least four nodule-enhanced SWEET transporters with predicted partially overlapping substrate specificities, the greatest challenge for elucidating their roles in SNF and AM is posed by possible functional redundancies, which may call for generation of double, triple, or even higher order mutants.

In our labs, we aim to characterize transporters and other gene products potentially involved in nodule iron metabolism. We have isolated *Tnt1* mutants for homologs of *LjMATE1* and *LjSEN1*. The *Medicago MATE* is not orthologous to *LjMATE1*, but it is also required for SNF. We have identified mutants for other iron-related nodule-specific genes, including a putative Fe^{3+}/Fe^{2+}-reductase, an Nramp transporter, a YSL transporter, and a ZIP transporter. Characterization of these mutants will help us understand iron homeostasis and metabolism, as well as roles of these transporters in SNF.

To date, no report has been published on putative Cu^+ transporters in nodules. We have obtained a *Tnt1* mutant for a nodule-specific channel from the CTR family, which is a candidate for Cu^+ transport. In addition, we have isolated *Tnt1* insertion alleles of a putative Zn^{2+} transporter gene from the CDF family. The synchrotron-based X-ray fluorescence technique provides subcellular resolution of metal partitioning and has been applied to nodulation studies only recently (Rodriguez-Haas et al., 2013). We are using this approach to visualize metals in mutants that are relevant to metal homeostasis in nodules.

82.11 CONSIDERATIONS ASSOCIATED WITH THE USE OF THE *Tnt1*-INSERTION MUTANT POPULATION

Because of superior *in vitro* regeneration and nodulation properties of *M. truncatula* ecotype R108 (Blondon et al., 1994; Hoffmann et al., 1997), it was chosen as the basis for the *Tnt1* mutant resource (Tadege et al., 2008; see Chapter 83). The genome sequencing and annotation efforts, however, have been conducted mostly on the related ecotype A17 (Young et al., 2011). Genetic divergence between these ecotypes presents a challenge for designing primers to be used in PCR-screening for *Tnt1* insertions in specific

genes. In many instances, the gene model is not identical between the two ecotypes. Thus, for each gene of interest, it is very important to determine the R108 gene model. A *Tnt1* insertion expected to inactivate a gene according to an A17-based gene model sometimes may cause no mutant phenotype. This can happen because the insertion is actually located outside of essential elements of the gene according to an R108-based model. Recently, as a part of the *Medicago* Hapmap project, the R108 genome has been sequenced (Stanton-Geddes et al., 2013), which should make reverse genetics using this genotype easier.

Although the *Tnt1*-insertion mutant population is a fairly new genetic resource, several genes have already been characterized using this population (see Chapter 83). Cloning of *MtNOOT* and *DNF2* are examples of how forward genetic screening of this population can lead to the discovery of new genes (Bourcy et al., 2013; Couzigou et al., 2012; see Chapter 49). Reverse genetics using this resource yielded the *MtRemorin* mutant and helped in the validation of map-based cloning of *MtIPD3* and *MtVPY* (Horvath et al., 2011; Lefebvre et al., 2010; Murray et al., 2011). Furthermore, 39 *Tnt1*-insertion alleles of known symbiotic genes have been identified in the *Tnt1*-insertion mutant population, demonstrating its power as a resource for gene discovery (Pislariu et al., 2012; see Chapter 83).

In a forward genetic screen, a *Tnt1* mutant with aborted IT phenotype was isolated and named *knocks but can't enter* (*KCE*). This phenotype was then linked with a mutation in *MtLIN* (Kiss et al., 2009). Interestingly, the mutation was not caused by a *Tnt1* insertion. A G-to-A transition at position 1740 in *MtLIN* introduced a premature stop codon (Guan et al., 2013). In line with the higher susceptibility of the R108 ecotype to stresses and colonization by rhizobia, both *ipd3* and *lin* mutants displayed a stronger phenotype in the R108 background compared to Jemalong ecotype A17. The point mutation that affected *MtLIN* in the *Tnt1* mutant illustrates possible consequences of the somatic embryogenesis-mediated regeneration process used as trigger for *Tnt1* remobilization, namely somaclonal variation. Although this phenomenon may impede attempts to clone genes causing mutant phenotypes in some forward genetics experiments, it will have little effect on reverse genetics experiments, especially if segregation analysis or genetic complementation confirms the link between genes and phenotypes.

The most probable effect of a *Tnt1* insertion is the inactivation of a gene (see Chapter 83). Five kinds of *Tnt1* mutants can be isolated from the population: (i) *Tnt1* insertion in an exon. This usually results in a complete loss of function or knockout (KO) phenotype. (ii) *Tnt1* insertion in the 3′ UTR. Such insertions can cause either complete KO (null mutant) or a KD phenotype, since it can destabilize the mRNA and reduce the expression of the gene. However, since the protein-coding region remains intact, it is possible that a smaller amount of the protein is produced, which might be sufficient to fulfill the necessary function. Disruption of the poly(A) tail resulting from *Tnt1* positioned in the 3′ UTR can have more profound effect on the transcript's fate. It is known from animal studies that interaction between poly(A)-binding protein and eukaryotic translation initiation factor 4G generates a circular structure important for recruiting the 43S ribosome assembly, which stimulates the 60S ribosomal subunit to join the 48S pre-initiation complex (Kahvejian et al., 2005; Searfoss et al., 2001). (iii) *Tnt1* insertion in the 5′ UTR. Such a *Tnt1* location can be associated with a wide range of phenotypes: from a very strong KO to no mutant phenotype. Several cases are known in which the 5′ UTR controls translation in a eukaryotic cell (Bugaut and Balasubramanian, 2012). Again, these examples involve mRNA destabilization and compromised translation. On the other hand, sometimes the 5′ UTR is not so crucial and/or alternative splicing can generate a functional mRNA, removing the insertion altogether and producing an unaltered phenotype. (iv) *Tnt1* inserted in a promoter region near the 5′ UTR. If the insertion is inside the TATA box, the most probable phenotype is a KO. However, if the promoter is disrupted outside of a major TF-binding site, a KD mutant phenotype could result. (v) *Tnt1* inside an intron. It may not cause a mutant phenotype, if the insertion is removed with the intron during transcript splicing, unless the 5-kb insertion interferes with transcription elongation. If the insertion coincides with an intron–exon junction, it is likely to cause a KO phenotype. Although these situations are theoretical scenarios, experimental evidence accumulated over the past few years of exploring the *Tnt1* population suggests examples for nearly all categories.

82.12 CONCLUSION

The *Medicago Tnt1* mutant population and the MtGEA are valuable resources for reverse genetic studies of the legume *M. truncatula*. Some 21,700 independent *Tnt1* insertion lines have been generated with probably over 500,000 insertions in the genome. This is predicted to cover ~90% of *Medicago* genes with an insertion. We took a systematic approach to isolate *Tnt1* mutants of nodule-enhanced TFs and transporters. The mutants recovered are a great platform for comprehensive functional studies on the corresponding genes and will significantly advance our knowledge about SNF.

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Chapter 83

Retrotransposon (*Tnt1*)-Insertion Mutagenesis in *Medicago* as a Tool for Genetic Dissection of Symbiosis in Legumes

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83.1 INTRODUCTION

The legume family (*Fabaceae*) of >18,000 species is the third largest among angiosperms and is second to cereals (*Poaceae*) as source of human food and animal forage and feed. Most legumes establish symbiotic associations with nitrogen-fixing bacteria (rhizobia), and by so doing, they contribute up to 40 million tons per year of "fixed" nitrogen to the soils they grow in (Peoples et al., 2009). This environmentally friendly interaction begins with a remote chemical signaling between compatible symbionts and culminates with the development of a new organ, the root nodule, where rhizobia reside and fix N_2 (Timmers et al., 1999). In response to flavonoid signals exuded by the legume roots, compatible rhizobia synthesize lipochito-oligosaccharide (LCO) signals known as nodulation (Nod) factors (NF) (D'Haeze and Holsters, 2002). Their perception by the *NOD FACTOR RECEPTOR 1*/*LYS-M DOMAIN-CONTAINING RECEPTOR-LIKE KINASE 3* (*NFR1*/*LYK3*) and *NFR5*/*NOD FACTOR PERCEPTION* (*NFP*) (see Chapter 51) triggers the infection signaling pathway that paves the way for rhizobial invasion and nitrogen fixation (Radutoiu et al., 2003; Limpens et al., 2003; Smit et al., 2007). Colonization starts with the attachment of rhizobia to root hair tips, followed by root hair deformation and curling, resulting in the entrapment of rhizobia in the so-called shepherd's crock (Fig. 83.1a). Local cell wall loosening, plasma membrane invagination, and the deposition of new cell wall material results in the development of a tube-like infection thread (IT), which serves as the path for rhizobia through several layers of plant cells toward the dividing inner cortical cells of the nodule primordium (NP) (Fig. 83.1b–d). Rhizobia are subsequently released from ITs into NP cells via an endocytosis-like process, encapsulated in a plant-derived membrane to become

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a new "organelle," the symbiosome (Brewin, 2004; Udvardi and Day, 1997). While nodule primordia continue to divide and grow into functional nodules, rhizobia differentiate, induce nitrogenase genes, and start fixing N_2. Reduced nitrogen from bacteroids and reduced carbon and other nutrients from the plant cytoplasm are transported across the symbiosome membrane for mutual benefit (Udvardi and Poole, 2013; see Chapter 68). In legumes of the robinoid subclade such as *Lotus*, bacteroids reach their mature state without changes in shape or size (Mergaert et al., 2006). In contrast, in legumes of the Inverted Repeat-Lacking Clade (IRLC) that include *Medicago*, *Cicer*, and *Pisum*, rhizobia undergo multiple rounds of endoreduplication en route to becoming the large, elongated, functional bacteroids (Van de Velde et al., 2010) (Fig. 83.1h). Host cells also undergo developmental reprogramming with different temporal layout among legumes, depending on the type of nodules they form: determinate or indeterminate. Legumes of the genera *Glycine*, *Phaseolus*, *Lotus*, and *Vigna* develop determinate, spherical nodules, whose meristem is lost shortly after their initiation, and their growth is solely a result of cell expansion. Members of the genera *Medicago*, *Trifolium*, *Pisum*, and *Vicia* form indeterminate, elongated nodules, which maintain an active apical meristem, display developmental and functional zonation, and continue to harbor functional bacteroids throughout their growth (Maunoury et al., 2007) (Fig. 83.1f).

Similar to 80% of vascular plants, legumes also establish symbiotic associations with arbuscular mycorrhizal (AM) fungi, which, in exchange for reduced carbon from the host, deliver phosphate, sulfur, nitrogen, and micronutrients into the host root cells (Harrison, 2012). Much like the nodulation symbiosis, mycorrhization starts with a signal exchange between the two partners: plant-released strigolactones and fungal mycorrhization (Myc) factors, triggering a symbiotic signaling cascade starting with Myc perception by *NFP* (Maillet et al., 2011). This process culminates with the intracellular development of highly branched fungal structures, arbuscules. Similar to nodulation, the nutrient exchange occurs at the interface between symbionts, the periarbuscular membrane (PAM) (Javot et al., 2007).

To gain insight into the genetic and molecular mechanisms governing the two symbioses, various bacterial, fungal, and plant model species have been used over the past few decades. Some agriculturally important legumes have complex polyploid or allopolyploid, and large genomes, and genetics can be difficult because some are out-crossing. The two selected model legumes are *Medicago truncatula* and *Lotus japonicus* (Handberg and Stougaard, 1992; Cook, 1999). They have relatively small genomes (~550 and ~470 Mbp, respectively), they are diploids, are in-breeding, and can be transformed by *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* (Chabaud et al., 2003). Several genetic resources have been developed for these two model legumes, including massive genome-wide expression repositories and mutant collections, which are instrumental for the fast pace discovery and functional characterization of key symbiotic genes. In this chapter, we present a summary of the *Medicago* and *Lotus* symbiotic genes and the mutagenesis methods used to characterize them functionally. In this context, we focus on the tobacco retrotransposon *Tnt1*-insertion *M. truncatula* mutant collection developed at the Samuel Roberts Noble Foundation (d'Erfurth et al., 2003; Tadege et al., 2008). With more than 21,000 independent insertion lines, this is the largest DNA-insertion mutant collection in legumes. From 9300 lines, we isolated 179 mutants displaying a range of defects in nodule development and function (Pislariu et al., 2012).

83.2 GENOMIC, TRANSCRIPTOMIC, AND MUTANT RESOURCES FOR THE FUNCTIONAL DISSECTION OF LEGUME SYMBIOSES

Legume interactions with both rhizobia and AM fungi involve extensive and coordinated transcriptional reprogramming in the symbiotic partners (Becker et al., 2004; Benedito et al., 2008; Verdier et al., 2013). Transcriptomic resources have been instrumental for the identification of key symbiotic genes (Table 83.1) and continue to lay the foundation for reverse genetics in legumes.

Both the *Medicago* and *Lotus* genomes have largely been sequenced, although the former is more complete (Young et al., 2011; Sato et al., 2008; see Chapter 78). Also, Illumina-Solexa technology is currently being used to resequence 384 *M. truncatula* ecotypes with at least 5× coverage and a subset of 30 lines with deep coverage (20× or more) to reveal single nucleotide polymorphisms (SNPs), insertions/deletions (INDELs), and copy number variants (CNVs) as part of the haplotype map (HAPMAP) project (Paape et al., 2012; Young and Bharti, 2012; see also Chapter 78). Linking common patterns of genetic variation with accession-specific phenotypes will enable genome-wide association mapping of agriculturally important traits such as seed nutrient composition, increased biomass production, and more efficient SNF and AM, which may be translated to crop legumes, to improve food production for example. Even though soybean is not a model legume, its genome has also been sequenced to completion (Schmutz et al., 2010), and transcriptomic resources are now available (Libault et al., 2010; Severin et al., 2010; see Chapter 41).

The development of mutant populations for forward genetics has been a priority for legume researchers over the past 10–15 years, and several mutant populations are now available for the functional characterization of plant genes involved in SNF, AM, and other processes. Chemical mutagenesis using ethyl methanesulfonate (EMS)

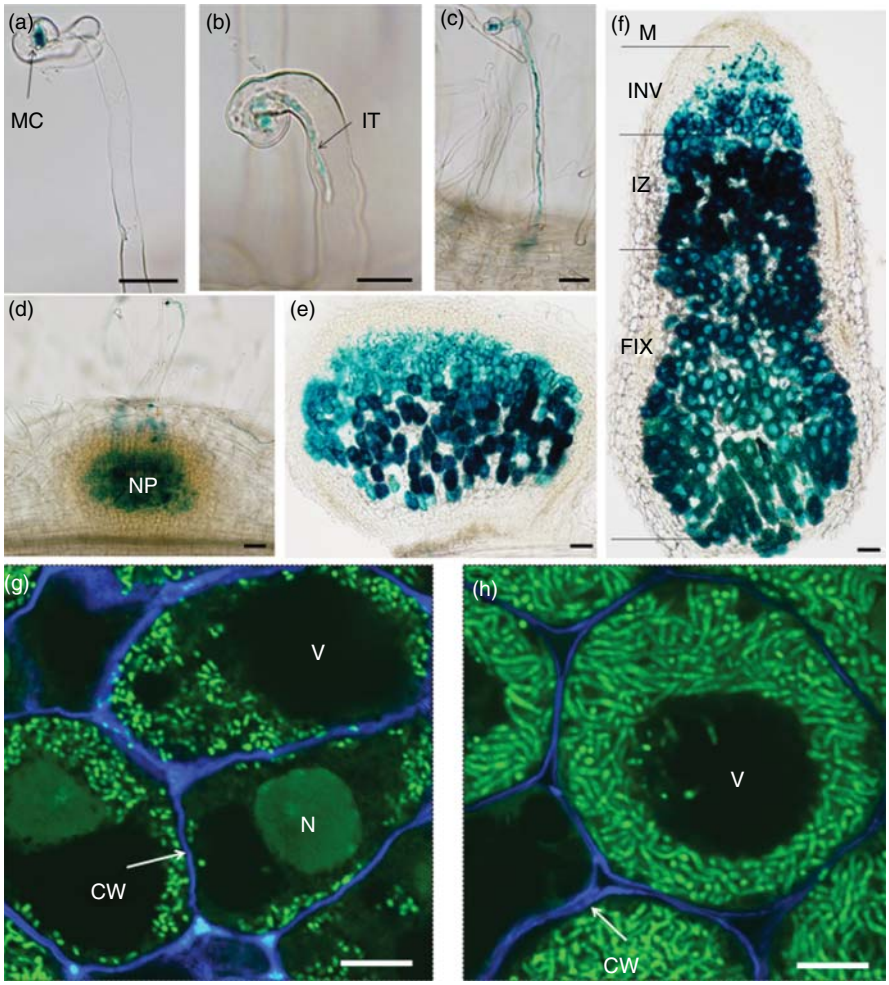


Figure 83.1 Nodule development in *Medicago truncatula*. *Sinorhizobium meliloti* carrying the *hemA*::*LacZ* reporter was used to infect *Medicago* roots. Samples harvested at 2 days postinoculation (DPI) (a, b, c), 4 DPI (d), 8 DPI (e), and 15 DPI (f) were stained with X-Gal to produce blue-stained rhizobia. Infection starts with root hair curling, entrapment of rhizobia, and development of a microcolony (MC) inside the curl (a). Along the root hair, an infection thread (IT) develops (b, c) and from this, rhizobia are released in the dividing cells of the nodule primordium (NP) (d). By 8 DPI, most cells of the emerging nodule are filled with rhizobia (e). The nodule continues to elongate and by 15 DPI, the nodule zonation including meristem (M), invasion zone (INV), interzone (IZ), and the nitrogen fixation zone (FIX) is visible. The same nodule sections were counterstained with the fluorescent DNA dye Syto13 and with calcofluor-white that stains cellulose and chitin in the cell walls. Rhizobia are shown as green fluorescence, and cell walls stained by calcofluor are seen in blue. Once inside host cells, rhizobia undergo differentiation from free-living-like small bacteria as seen in the invasion zone (g) to elongated, rod-shaped bacteroids, as seen in the nitrogen-fixation zone (h). Abbreviations: V, vacuole; N, nucleus; CW, cell wall. Scale bars: 100 µm (a, c, d, e, and f); 50 µm (b) and 10 µm (g, h).

induces random point mutations and has been used successfully in *Medicago* (Benaben et al., 1995; Penmetsa and Cook, 2000; see Chapter 63). Molecular and genetic characterization of EMS mutants defective in nodule initiation and development, nodule number, and/or AM symbiosis led to the map-based cloning of 13 key symbiotic genes in *Medicago*: Nod factor receptor *LYK3*; the Nod-factor and Myc receptor *NFP*; the common SNF and AM symbiotic pathway genes *DOESN'T MAKE INFECTIONS 1*, *2*, *3* (*DMI1*, *DMI2*, *DMI3*); *INTERACTING PROTEIN OF DMI3* (*IPD3*); *NODULATION SIGNALING PATHWAY 2* (*NSP2*); SNF-specific transcription factors *ERF REQUIRED FOR NODULATION 1* (*ERN1*) and *NODULATION SIGNALING PATHWAY 1* (*NSP1*); genes involved in infection, *NUMEROUS INFECTIONS AND POLYPHENOLICS/LATERAL ROOT ORGAN DEFECTIVE* (*NIP/LATD*), *RHIZOBIUM-DIRECTED POLAR GROWTH* (*RPG*); and *SICKLE* (*SKL*) and *SUPER NUMERIC NODULES* (*SUNN*) that control the extent of nodulation (Table 83.1). EMS mutagenesis has also been applied successfully to *Glycine* (Carroll et al., 1985) and *Lotus* (Szczyglowski et al., 1998). Symbiotic genes *NFR1*; *NFR5*; ion channels *CASTOR* and *POLLUX*; the *SYMBIOSIS-RELATED KINASE* (*SYMRK*); the *Ca2+/CALMODULIN-DEPENDENT KINASE CCaMK*; *CYCLOPS*; *LjNSP1*; and *LjNSP2* were positionally cloned using *Lotus* EMS populations (Table 83.1). Molecular cloning of soybean symbiotic genes has been less successful due to the higher complexity of its larger, paleotetraploid genome, and is most commonly approached by taking advantage of the syntenic relationship with *Lotus* (Indrasumunar et al., 2010).

Ionizing radiation in the form of gamma rays and fast neutrons induces deletions and other chromosomal rearrangements, and often generates complete loss-of-function or knockout (KO) mutants. The first *Medicago* symbiotic mutants were isolated from a gamma-ray mutagenized population (Sagan et al., 1995). Among these were deletion alleles of *DMI2*, *DMI3*, and *SUNN* (Table 83.1). Large, fast-neutron bombardment (FNB) mutant collections have been generated at The Samuel Roberts Noble Foundation in the United States and at the John Innes Center in the United Kingdom

Table 83.1 Symbiotic Genes and Mutagenesis Approaches Used to Characterize Their Biological Functions in Medicago and Lotus

Gene (Protein)	*Tnt1*-Insertion Alleles	Symbiotic Phenotype and Type of Mutagenesis (*Medicago*/*Lotus*)	References
Transcription Factors			
MtbHLH476 (bHLH)	*Tnt1*-Jemalong	Fewer nodules (RNAi and *Tnt1*)	Ariel et al. (2012)
MtbHLH1 (bHLH)	*Tnt1* lines available (Sinharoy et al., u.d.)	Aberrant nodule vasculature (CRES-T)	Godiard et al. (2011)
MtEFD (AP2-ERF)	NF4401; NF4919	Inf++; Nod++Fix− (RNAi, FNB)	Vernie et al. (2008)
LjERF1 (AP2-EREBP)	*Tnt1* lines available (Sinharoy et al., u.d.)	Fix+/− (RNAi)	Asamizu et al. (2008)
MtERN1 (*AP2-ERF*)	NF1390; **NF5040**	Nod− (FNB; EMS; *Tnt1*)	Middleton et al. (2007)
MtERN2 (*AP2-ERF*)			Andriankaja et al. (2007)
MtERN3 (*AP2-ERF*)			Andriankaja et al. (2007)
MtHAP2-1/*LjNF-YA1* (NF-YA)		Nod+/− (delayed nodulation) Fix− (RNAi/RNAi)	Combier et al. (2006); Soyano et al. (2013)
MtIPD3/*LjCYCLOPS* (coiled-coil domain)	NF5939	Nod+Fix+; Nod+Fix− (EMS,FNB, *Tnt1*)	Yano et al. (2008); Horvath et al. (2011)
MtNIN/*LjNIN* (RWP-RK)	NF2728; NF0532; NF1317 NF1277; NF1263; NF3019 NF0117; NF3046; NF2640 NF0440; NF0825; NF2700 Tnk148; **NF1422; NF4257 NF5802; NF5855**	Nod− (FNB; *Tnt1*/T-DNA)	Marsh et al. (2007); Schauser et al. (1999)
MtNOOT (BTB/POZ-ankyrin repeat)	NF4445; NF5894; NF2717 Tnk507	Emergence of roots from the nodule apex (*Tnt1*)	Couzigou et al. (2012)
MtNSP1/*LjNSP1* (GRAS)	NF1594; NF0848; NF2393	Nod− (EMS; *Tnt1*/EMS)	Smit et al. (2005); Heckmann et al. (2006)
MtNSP2/*LjNSP2* (GRAS)	NF0208; NF0814; NF0584 NF2811; NF0351; **NF4839**	Nod− (EMS; *Tnt1*/EMS)	Kalo et al. (2005); Heckmann et al. (2006)
LjnsRING (RING H2-Finger)		Nod− (RNAi)	Shimomura et al. (2006)
MtRR9; *MtRR11* (RR-A-type)		Reduction in nodule primordium (RNAi)	Op den Camp et al. (2011)
LjSIP1 (ARID)		Reduced nodulation Fix− (RNAi)	Wang et al. (2013)
Protein Trafficking			
MtDNF1 (SPC)		Nod+Fix− (FNB)	Wang et al. (2010)
MtRab7 (RAS)		Nod+Fix− (RNAi)	Limpens et al. (2009)
LjSYP71 (SNARE)		Fix− (EMS, CIB)	Hakoyama et al. (2012b)
MtSYP132 (syntaxin)			Limpens et al. (2009)
MtVAMP721d/e (VAMP)		Fix− (RNAi)	Ivanov et al. (2012)
MtVPY (vapyrin)	NF6898; NF4489; **NF5844**	Nod− (FNB; *Tnt1*)	Murray et al. (2011)
Kinases and Kinase-Interacting Proteins			
MtCDC16 (APC/C)		Nod++ (RNAi)	Kuppusamy et al. (2009)
MtCDPK1 (CDPK)		Nod+/− (RNAi)	Ivashuta et al. (2005)
LjCIP73 (CCaMK-interacting protein)		Fix− (RNAi)	Kang et al. (2011)
MtCPK3 (CDPK)		Nod++ (RNAi)	Gargantini et al. (2006)
MtCRE1/*LjLHK1* (HK)		Nod+/− (RNAi/RNAi)	Gonzalez-Rizzo et al. (2006); Murray et al. (2007)
MtDMI2/*LjSYMRK* (LRR-RLK)	NF1449; NF8854	Nod−; release defect in RNAi plants (EMS; γ-rays; *Tnt1*; RNAi/EMS)	Endre et al. (2002)
MtDMI3/*LjCCaMK* (CCaMK)	NF0028; NF0692; NF0600 NF1757	Nod− (EMS; γ-rays; *Tnt1*)	Levy et al. (2004); Tirichine et al. (2006)

Table 83.1 (*Continued*)

Gene (Protein)	*Tnt1*-Insertion Alleles	Symbiotic Phenotype and Type of Mutagenesis (*Medicago*/*Lotus*)	References
Kinases and Kinase-Interacting Proteins			
MtHMGR1 (HMGR)		Nod− (RNAi)	Kevei et al. (2007)
MtIRE (AGC kinase)	*Tnt1* lines available (Veerappan et al., u.d.)		Pislariu and Dickstein (2007)
MtLYK3 (*hcl*)/*LjNFR1* (LysM-RLK)	**Tnk387-LTR4**	Nod+/− (EMS; RNAi, *Tnt1*)	Limpens et al. (2003); Radutoiu et al. (2003)
MtNFP/*LjNFR5* (LysM-RLK)		Nod− (EMS; RNAi)	Arrighi et al. (2006); Madsen et al. (2003)
MtPI3K (PI3K)			Peleg-Grossman et al. (2007)
MtPUB1 (E3 ligase)		Fix− (EE)	Mbengue et al. (2010)
LjSINA (E3 ligase)		Fix− (EE)	Den Herder et al. (2012)
LjSIP2 (SymRK-interacting protein)		Nod+/− (RNAi)	Chen et al. (2012)
SUNN (CLV1-like LRR RK)	NF1526; NF1858; NF0984 NF3352; NF1709; NF2629 Tnk100; **NF5448; NF5814**	Nod++ (EMS; γ-rays; *Tnt1*)	Schnabel et al. (2005)
MtSYMREM1/*LjSYMREM1* (remorin)	NF3495; NF4432	Nod+Fix− (RNAi; *Tnt1*)	Lefebvre et al. (2010)
Calcium Pores and Channels			
MtDMI1/*LjCASTOR*; *LjPOLLUX* (ion channel)	NF0359; NF0493; NF3128	Nod− (EMS; *Tnt1*/EMS)	Ane et al. (2004); Imaizumi-Anraku et al. (2005)
MtMCA8 (SERCA ATPase)		Nod+Fix+ (RNAi)	Capoen et al. (2011)
LjNENA (Sec13/Seh1)		Fix+/− (EMS)	Groth et al. (2010)
LjNUP85 (nucleoporin)		Nod− (EMS)	Saito et al. (2007)
LjNUP133 (nucleoporin)		Nod+/− (fewer nodules) (T-DNA)	Kanamori et al. (2006)
Transporters and Enzymes			
MtCHR (chalcone reductase)		Nod+/− (RNAi)	Zhang et al. (2009)
MtCHS (chalcone synthase)		Nod− (RNAi)	Zhang et al. (2009)
MtDNF2 (PLC)	NF0217; NF2496	Nod+Fix− (FNB; *Tnt1*)	Bourcy et al. (2013)
MtENOD8 (esterase)			Pringle and Dickstein, (2004)
LjFEN1 (homocitrate synthase)			Hakoyama et al. (2009)
MtFNSII (flavone synthase II)		Nod+/− (fewer nodules) (RNAi)	Zhang et al. (2007)
MtγECS (GSL)		Reduced nodule size and SNF efficiency (RNAi)	El Msehli et al. (2011)
MtGSNI (GSI)			Carvalho et al. (2003)
LjMATE1 (citrate transporter)	*Tnt1* lines available (Kryvoruchko et al., u.d.)	Fix− (RNAi)	Takanashi et al. (2013)
MtMMPL1 (*MtN9*) (metalloproteinase)		Nod+Fix+ (RNAi)	Combier et al. (2007)
MtNIP (*MtLATD*) (nitrate transporter)		Nod+Fix− (EMS)	Yendrek et al. (2010)
MtNOA-RIF1 (GTPase)		Reduced nodulation and SNF efficiency (RNAi)	Pauly et al. (2011)
LjNPL (pectate lyase)		Nod+Fix− (EMS)	Xie et al. (2012)
MtPT1 (phosphate transporter)			Liu et al. (2008)
MtPT2 (phosphate transporter)			Liu et al. (1998)
MtRbohA (NADPH oxidase)		Nod+Fix+/− (RNAi)	Marino et al. (2011)
MtRIP1 (peroxidase precursor)			Peng et al. (1996)
LjSEN1 (VIT)	*Tnt1* lines available (Kryvoruchko et al., u.d.)	Fix− (EMS, T-DNA)	Hakoyama et al. (2012a)

(*continued*)

Table 83.1 (*Continued*)

Gene (Protein)	*Tnt1*-Insertion Alleles	Symbiotic Phenotype and Type of Mutagenesis (*Medicago*/*Lotus*)	References
Transporters and Enzymes			
LjSST1 (sulfate transporter)	*Tnt1* lines available (Kryvoruchko et al., u.d.)	Fix− (T-DNA, EMS)	Krusell et al. (2005)
MtSucS1/*LjSUS1*, *SUS3* (SuSy)		Nod+/−Fix+/− (antisense/TILLING)	Baier et al. (2007); Horst et al. (2007)
MtTRX S1 (thioredoxin S1)			Alkhalfioui et al. (2008)
MtTRX S2 (thioredoxin S2)			Alkhalfioui et al. (2008)
Small Peptides			
MtENOD2 (proline-rich)			van de Wiel et al. (1990)
MtENOD11 (proline-rich)			Journet et al. (2001)
MtENOD12 (proline-rich)			Pichon et al. (1992)
MtDVL1 (devil protein)		Nod+/− (fewer nodules, aborted ITs) (OX)	Combier et al. (2008)
MtNOD25 (nodulin)			Hohnjec et al. (2009)
MtPR-1 (pathogenesis-related)			Szybiak-Strozycka et al. (1995)
MtPRP4 (proline-rich)			Wilson et al. (1994)
MtRAFL1 (rapid alkalinization factor)		Nod+/− (fewer nodules, aborted ITs) (OX)	Combier et al. (2008)
MtSNARP (RNA-binding)		Nod+Fix− and aberrant nodule structure (RNAi)	Laporte et al. (2010)
Hormone-Related			
MtPIN2 (auxin efflux carrier)	**NF5786; NF2784**	Nod+/− (fewer nodules) (RNAi)	Huo et al. (2006)
MTPIN3 (auxin efflux carrier)		Nod+/− (fewer nodules) (RNAi)	Huo et al. (2006)
MtPIN4 (auxin efflux carrier)		Nod+/− (fewer nodules) (RNAi)	Huo et al. (2006)
MtSKL (ethylene insensitive)	NF2085	Nod++ (EMS; *Tnt1*)	Penmetsa et al. (2008)
MtSTA (*sensitivity to ABA*)		Nod+/− (FNB)	Ding et al. (2008)
Others			
MtANN1 (annexin)			Carvalho Niebel et al. (1998)
MtENOD16 (phytocyanin-related)			Greene et al. (1998)
MtENOD20 (phytocyanin-related)			Greene et al. (1998)
MtENOD40 (nontranslatable RNA)		Nod+/− (fewer nodules) Fix− (RNAi)	Wan et al. (2007)
MtFLOT2 (flotillin)		Nod+/− (fewer nodules) (RNAi)	Haney and Long, (2010)
MtFLOT4 (flotillin)		Nod+/− (fewer nodules) (RNAi)	Haney and Long, (2010)
LjIGN1 (ankyrin repeat TM protein)		Fix− (SMC)	Kumagai et al. (2007)
MtLIN/*LjCERBERUS* (U-Box/WD40)		Nod− (EMS)/(Ac, EMS, T-DNA)	Kiss et al. (2009); Yano et al. (2009)
MtN1 (Cys-rich pathogen-inducible)			Gamas et al. (1998)
MtN5 (lipid-transfer protein)		Nod+/− (fewer nodules) Fix+ (RNAi)	Pii et al. (2012)
MtN6 (similarity with *Emericella nidulans* FluG)			Gamas et al. (1996)
MtN12 (extensin-like)			Gamas et al. (1996)
MtN13 (PR-10-like)			Gamas et al. (1998)
MtRBP1 (RNA-binding protein)			Campalans et al. (2004)
MtRDN1 (unknown)	**NF4620**	Nod++ (FNB)	Schnabel et al. (2011)
MtRIT1 (*NAP1*)/*LjNAP1* (SCAR/WAVE)		Nod− (FNB/T-DNA)	Miyahara et al. (2010); Yokota et al. (2009)
MtRPG (coiled-coil protein)		Nod− (EMS)	Arrighi et al. (2008)

Mt, *Medicago truncatula*; Ms, *Medicago sativa*; Lj, *Lotus japonicus*; SMC, Somatic mutation through intensive culture; CIB, carbon-ion beams; EE, ectopic expression; CRES-T, Chimeric REpressor Silencing Technology; OX, Overexpression; u.d., unpublished data.

Tnt1-insertion lines with flanking sequence tags (FSTs) identified by sequencing DNA pools representing the entire *Tnt1*-insertion population and unconfirmed at the level of individual plant are shown in bold font. Shaded in gray are genes with no mutants described at this time. Due to space limitation, a selection of references is shown.

(Wang et al., 2006). From these collections, transcript-based cloning was used to identify deletions affecting the following symbiotic genes: *ERN1*; *DMI1*; *DMI3*; *NSP2*; *NODULE INCEPTION* (*NIN*); *DEFECTIVE IN NITROGEN FIXATION* (*DNF1*); *ETHYLENE-RESPONSIVE FACTOR REQUIRED FOR NODULE DIFFERENTIATION* (*EFD1*); *REQUIRED FOR INFECTION THREAD* (*RIT1*); *VAPYRIN* (*VPY*); and *SENSITIVITY TO ABA* (*STA1*) symbiotic genes (Table 83.1). Despite some success, transcript-based cloning is not trivial. The recently developed *M. truncatula* genome-wide tiling microarray by NimbleGen may speed up gene discovery in deletion mutant populations in the future, although no novel symbiotic gene has been cloned so far using this approach.

When expressed sequence tag (EST) libraries became available (Gyorgyey et al., 2000), RNA-induced gene silencing or RNA-interference (RNAi) was developed to characterize functionally genes of interest in a reverse genetics approach (Limpens et al., 2003). Even though it is not a high-throughput method, it requires plant transformation and tissue culture, and transcript reduction is highly variable (phenotypes ranging from wild-type-like to KO), RNAi has been used to characterize at least 20 symbiotic genes for which no mutants were available (Table 83.1). Among these are the *Ca^{2+}-DEPENDENT PROTEIN KINASE 1* (*CDPK1*); *FLOTILLINS FLOT2* and *FLOT4*; *CYTOKININ RECEPTOR CRE1*; *EARLY NODULIN ENOD40*; *3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE 1* (*HMGR1*); *MATRIX METALLOPROTEINASE-LIKE 1* (*MtMMPL1*); *LIPID TRANSFER PROTEIN* (*LTP*, *MtN5*); and *PIN-FORMED AUXIN EFFLUX CARRIERS PIN2*, *PIN3*, *PIN4*. Another reverse genetics approach that has emerged recently is TILLING (targeting-induced local lesions in genomes), which allows PCR-based identification of allelic series using EMS-mutagenized collections (Le Signor et al., 2009; Perry et al., 2003). In combination with next-generation sequencing (NGS), TILLING has the potential for high-throughput assays by multiplexing gene targets and genomes. Recently, two nuclease-based approaches to introduce genomic modification have been tested: zinc finger nucleases (ZFNs) (Curtin et al., 2011) and transcription activator-like effector nucleases (TALENs) (Kay and Bonas, 2009). It is plausible that such methods will revolutionize engineering of crop genomes, mainly because of their remarkable specificity and because most modifications are not likely to be regulated as transgenic, thus facilitating widespread improvement of crop quality. For the time being, however, discovering biological functions for many open-reading frames (ORFs) of unknown function revealed by genome-sequencing projects, using mutant collections, remains a powerful and straightforward approach. The development of DNA-insertion mutagenesis as a tool for linking visible mutant phenotypes with new gene functions is beginning to increase the pace of gene discovery in model legumes.

T-DNA, transposons, and retrotransposons are excellent tools for disrupting genes to generate loss-of-function mutants. The mutated genes can quickly be identified by PCR, using the inserted DNA sequence as a "tag." T-DNA tagging has been very successfully applied in *Arabidopsis thaliana* because of the small plant size, ease of *Agrobacterium*-mediated transformation, and possibility to regenerate enormous number of plants in a short period of time (Clough and Bent, 1998). However, this method had limited success in legumes because they are larger, are not as easy to transform, and the time frame between transformation and seed harvest is between 7 and 12 months. DNA transposons translocate to new positions within genomes via a "cut-and-paste" mechanism, and because of their tendency to reinsert in the proximity of their original location, multiple rounds of transformation are required for good coverage and eventual saturation mutagenesis (Scholte et al., 2002). Transposons that have been tested in legumes are the maize (*Zea mays*) *Ac*/*Dc* system in *Lotus* (Thykjaer et al., 1995) and soybean (Mathieu et al., 2009), the *En*/*Spm* and *Tag1* systems in *Medicago* (d'Erfurth et al., 2006), and the rice miniature inverted repeat transposable element *MITE mPing* element in soybean (Hancock et al., 2009). However, they also tend to transpose into A/T-rich regions, often in intergenic spaces, and can be excised, resulting in the loss of tag from mutated genes, but leaving behind INDEL footprints, which makes them less suitable for genome-wide mutagenesis. These elements also show rapid inactivation when introduced into heterologous genomes, probably as a result of silencing, making them not usable for large-scale mutagenesis. On the other hand, the mPing element continues to transpose during normal plant growth, thus causing more mutations that may complicate genotyping and phenotyping of progeny.

Retrotransposons are mobile elements that transpose via a "copy-and-paste" mechanism through an intermediate mRNA, which is transcribed to cDNA and integrated at random in the genome, albeit preferentially in gene-rich regions within the genome (Amar et al., 1999). Transposition occurs during tissue culture, and, unlike DNA transposable elements, it causes stable mutations that are passed onto progeny during seed-to-seed propagation (d'Erfurth et al., 2003). Several retrotransposons have already been characterized in legumes and proved to be useful insertion mutagens in both autologous and heterologous systems. They include the LOtus REtrotransposon 1 (*LORE1a*), an exon-targeting endogenous member of the "*Gypsy*" superfamily, which has been shown to transpose in male germline (Madsen et al., 2005); the MEdicago RetroElement 1–1 (*MERE1-1*) in *Medicago*, which was identified as an insertion in the *NSP2* symbiotic gene (Rakocevic et al., 2009); and the *Cicer arietinum* retro-element 1 (*CARE1*), a *TY3-Gypsy*-like long terminal repeat LTR retroelement in chickpea (Rajput and Upadhyaya, 2010). LTR retrotransposons from tobacco (*Tnt1* and *Tto1*) and rice (*Tos17*) have been used to generate insertional mutants in *Arabidopsis* (Okamoto and Hirochika, 2000) and in *Medicago* (d'Erfurth et al., 2003; Tadege et al., 2008). A concerted effort by European and US researchers to implement *Tnt1*-insertion mutagenesis in

Medicago resulted in the largest collection of DNA-insertion mutants of all legumes (d'Erfurth et al., 2003; Tadege et al., 2008).

83.3 ISOLATION OF *TNT1*-INSERTION MUTANTS WITH DEFECTS IN NODULE DEVELOPMENT AND SYMBIOTIC NITROGEN FIXATION

Approximately 21,000 independent insertion lines have been generated at The Samuel Roberts Noble Foundation by somatic embryogenesis using leaf explants from a starter line, Tnk88-7-7 containing approximately five *Tnt1* copies that were transferred to wild-type R108 by *A. tumefaciens*-mediated transformation (Fig. 83.2). Approximately 10,500 lines have been preliminarily screened so far for visible phenotypes during community screening workshops, and 11,544 photographs are currently available at the mutant database (http://medicago-mutant.noble.org/mutant/). Approximately 30% of all screened lines displayed developmental, morphological, and symbiotic phenotypes, as reported earlier (Tadege et al., 2008). *Tnt1* was also deployed into the Jemalong 2HA ecotype as part of the European Union-Grain Legumes Integrated Project (EU-GLIP) (Iantcheva et al., 2009). Currently this European collection consists of approximately 5000 insertion lines from which nearly 2000 flanking sequence tags (FSTs) have been recovered.

Since 2006, the Noble Foundation has been hosting annual community screening workshops where researchers have the opportunity to identify, first-hand, *Tnt1*-insertion mutants with phenotypes of interest to them. In most years, the *Tnt1*-insertion populations were coinoculated with wild-type rhizobia and AM fungi to identify nodulation and

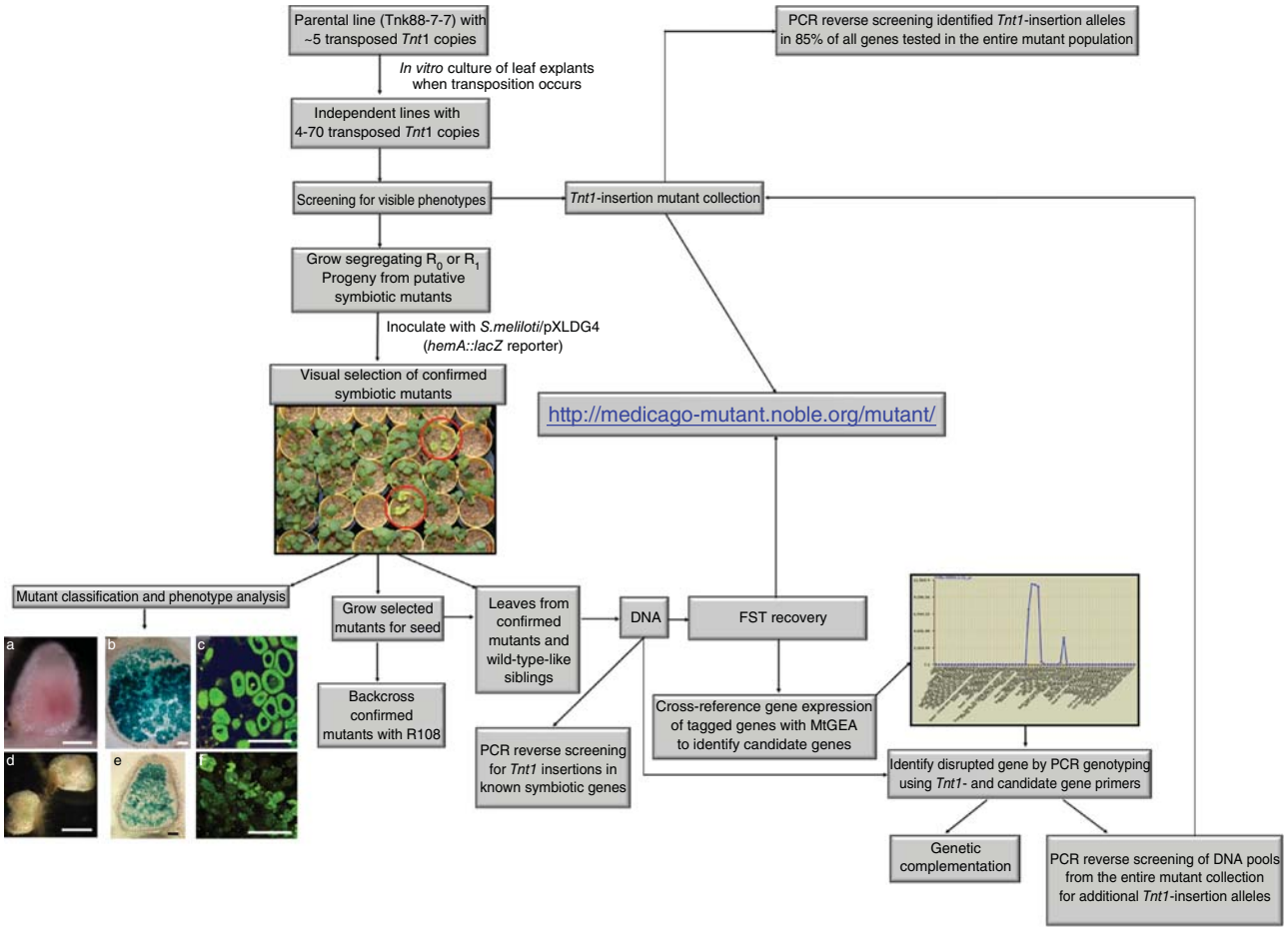


Figure 83.2 Flowchart detailing the establishment of the tobacco retrotransposon *Tnt1*-insertion mutant population in *Medicago truncatula* and steps taken to identify disrupted genes by forward and reverse genetics. Nodule phenotypes are shown for wild type (a, b, and c) and NF0440 (d, e, and f). Images represent whole mounts (a and d), 50-µm-section stained with X-Gal (b and e), and Syto-13-stained sections imaged by confocal microscopy (c and f). Scale bars: 2 mm (a); 1 mm (d); 100 µm (b, c, e, and f).

mycorrhization mutants. Approximately 1500 lines were screened every year, and a wide array of phenotypes has been reported (Tadege et al., 2008). From 317 lines with putative SNF phenotypes, which were identified after screening 9300 lines, 230 were rescreened in the presence of *Sinorhizobium meliloti* Sm2011, and symbiotic phenotypes were confirmed in 156 lines. *Rhizobium*-inoculated plants were analyzed at 15 and 21 days postinoculation (DPI), when wild-type R108 displayed healthy shoots with dark green leaves and large, functional, pink nodules. Generally, plants impaired in nodule development or defective in nitrogen fixation display stunted growth and are easily distinguished due to their nitrogen-deficiency phenotypes (increased anthocyanin accumulation in the shoot and leaf chlorosis/yellowing) (Fig. 83.2). From 156 lines, 179 SNF mutants were isolated; in some instances, multiple symbiotic mutants were found in a single line, presumably due to tagging of multiple symbiotic genes. The phenotypes were classified into six categories and included 72 Nod− (roots devoid of nodules); 51 Nod+Fix− (nodules develop but generally fail to accumulate leghemoglobin and rhizobia they harbor do not fix nitrogen); 17 Nod+Fix+/− (nodules are pale pink and rhizobia fix nitrogen less efficiently); 27 Nod+/−Fix− (small, ineffective bumps), 1 dNod+/−Fix+ (nodules develop later than in wild type but eventually develop into nitrogen-fixing organs), and 11 Nod++Fix+/− (larger number but less effective nodules than wild type) (Fig. 83.3). Symbiotic phenotypes were analyzed in detail, by a combination of stereo, bright field, and confocal microscopy. *S. meliloti* Sm2011 carrying the *hemA*::*LacZ* reporter was used for inoculation; histochemical staining with X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside), enabled rhizobia to be seen via blue precipitate (Figs. 83.1 and 83.2). Nod- and Nod+Fix− mutants were described in detail and unusual, potentially new phenotypes were highlighted (Pislariu et al., 2012). Among the latter are mutants isolated from lines NF0438, NF0359, NF5794, NF5654, NF0063, NF2496, NF4608, NF4928, NF1320, NF0440, and NF0134.

To assess the degree of rhizobial differentiation into bacteroids in Nod+Fix− mutants, the same nodule sections were counterstained with the fluorescent DNA dye Syto13, shown as green fluorescence (Figs. 83.1, 83.2, and 83.4). In wild-type nodules, functional bacteroids appear as elongated, rod-shaped organelles (symbiosomes), packing the cytoplasm of infected cells (Figs. 83.1h and 83.4a). Incomplete bacteroid differentiation, as suggested by the shorter rod-like appearance, was observed in numerous Fix- nodules such as those isolated from NF0134, NF2496, and NF4608 (Fig. 83.4c, g, h) and in many Fix+/− nodules, for example, in NF0306 and NF1858 (Fig. 83.4e, f). In some mutants, bacteroids undergo early degradation (NF1858, NF2496, and NF4608). An example of rhizobia that do not differentiate and remain small, similar to the free-living form, is the mutant identified in line NF0235 (Fig. 83.4d). In some mutants, the nodules appear almost empty, with sparse ITs from which rhizobia are released, but remain undifferentiated. Such an example is the mutant isolated from line NF0063 (Fig. 83.4b).

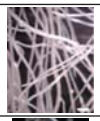
Symbiotic phenotype	Number of symbiotic mutants	Representative phenotype
Nod–	72	
Nod+ Fix–	51	
Nod+ Fix+/–	17	
Nod+/- Fix–	27	
dNod+/– Fix+	1	
Nod++ Fix+/–	11	
Wild type		
Total	179	

Figure 83.3 Classification of the symbiotic *Tnt1*-insertion mutants generated in the *Medicago* R108 ecotype. Representative phenotypes for each category are depicted. Images were acquired using an Olympus SZX12 stereomicroscope equipped with a Nikon DXM1200C digital camera. Scale bars: 1 mm.

Overall, this collection consists of mutants displaying a wide array of symbiotic phenotypes, some similar to those of known symbiotic mutants, other displaying unique features.

83.3.1 *Tnt1*-Insertion Alleles of Known Symbiotic Genes

In order to establish the *Medicago Tnt1*-insertion mutant collection as a primary resource for forward and reverse genetics among legumes, efficient sequencing of the genomic regions adjacent to the sites of *Tnt1* integration is imperative. Thermal asymmetric interlaced-PCR (TAIL-PCR) (Cheng et al., 2011), touchdown-PCR, and inverse-PCR (Ratet et al., 2010) have been used to recover

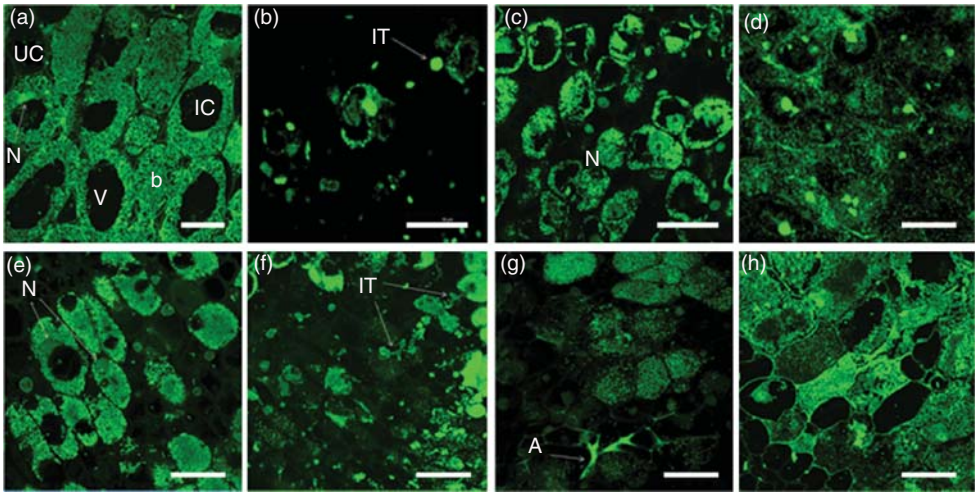


Figure 83.4 Symbiotic phenotypes of *Tnt1*-insertion mutants. Nodule sections of 50 µm thick were stained with the green fluorescent DNA dye Syto13 and imaged using confocal microscopy. In R108 wild-type nodules (a) rhizobia fill up the host cell cytoplasm and they differentiate into rod-shaped, elongated, nitrogen-fixing bacteroids (b). Panels (b–h) depict representative images of symbiotic phenotypes in NF0063, NF0134, NF0235, NF0306, NF1858, NF2496, and NF4608. Abbreviations: V, vacuole; IT, infection threads; A, autofluorescence; N, nuclei; UC, uninfected cell; IC, infected cell. Scale bars = 50 µm.

these so-called FSTs. To date, 44,238 FSTs have been sequenced and added to the searchable mutant database (http://medicago-mutant.noble.org/mutant/). Between 4 and 70 FSTs have been retrieved from each line sequenced, leading to an estimate of more than 525,000 insertions in the entire mutant population.

To assess the extent of mutagenesis in the *Tnt1*-insertion population, screening for insertions in known genes that cause either Nod− or Nod++ phenotype when mutated was carried out on mutants with confirmed Nod− and Nod++ phenotypes. FST sequencing and PCR reverse screening using combinations of gene-specific and *Tnt1*-specific primers followed by sequencing were used to identify mutants with insertions in the following genes: *NFP*, *DMI1*, *DMI2*, *DMI3*, *ERN1*, *NSP1*, *NSP2*, *NIN*, *LYK3*, *NUCLEOPORINS* (*NUP85* and *NUP133*), *SKL*, and *SUNN*. In the case of *NFP*, *LYK3*, *NUP85*, and *NUP133*, no insertion alleles were identified. However, between 1 and 13, alleles were identified in the other 9 genes tested: 3 for *DMI1*, 2 for *DMI2*, 4 for *DMI3*, 1 for *ERN1*, 3 for *NSP1*, 5 for *NSP2*, 13 for *NIN*, 1 for *SKL*, and 7 for *SUNN* (Pislariu et al., 2012). A total of 31 *Tnt1* insertions in known nodulation genes were found in Nod− mutants, which means that novel nodulation genes may be tagged in the remaining 41 Nod− mutants. Alternatively, it is possible that during tissue culture, which is used to trigger *Tnt1* retrotransposition, other endogenous DNA transposons may have been activated (Rakocevic et al., 2009), or single base pair changes may have been induced (Guan et al., 2013). Consequently, the mutant collection may contain some non-*Tnt1*-insertion alleles of known symbiotic genes. A protocol for recovering MERE1 FSTs has been developed and may help identify such non-*Tnt1*-insertion alleles. Nevertheless, several Nod− phenotypes appear to be unlike any of the known Nod− mutants, so it is expected that novel nodulation genes will be identified among the remaining 41 Nod− mutants.

In line with the previously reported preference of *Tnt1* to insert in gene-rich regions, and especially in exons, 80% of insertions identified in the nine genes tested were exonic, 10% were intronic, and 10% were either in the 5′ or the 3′ untranslated regions (UTRs). Because of this feature, many *Tnt1* insertions cause KO mutations. From a total of 39 insertion alleles, 4 were retrieved by FST sequencing only, 25 were identified by PCR reverse screening, and 10 by using both methods. Despite the fact that this was a small-scale experiment and was conducted mainly to demonstrate the reliability of the mutant collection, it is clear that most insertion alleles were identified by PCR reverse screening, highlighting the moderate performance of TAIL-PCR-based FST identification. Currently, we are developing cost-effective high-throughput means to recover FSTs by Illumina sequencing of DNA pools. We have tested 10×10 (for 100 lines) and 20×20 (for 400 lines) 2D pooling methods with pool-specific 7-base bar-codes in the primers for the second-round TAIL-PCR. In two pilot runs, we have achieved satisfactory results. The resulting sequences are regularly added to the mutant database.

Screening for AM phenotypes in 39 Nod− mutants showed that 25 of these could not establish AM symbiosis (Myc−), 5 mutants had less severe defects (Myc+/−), and 9 mutants were not defective (Myc+). Among these were 10 mutants in which *Tnt1* disrupted common symbiotic genes *DMI1*, *DMI2*, *DMI3*, and *NSP2*, and displayed the expected Myc− phenotypes. Some of the *nin* insertion

alleles had Myc− phenotypes, which is not consistent with the *nin* mutant phenotype that is limited to nodulation, and may therefore be due to additional insertions in unknown mycorrhization genes. The combined nodulation and mycorrhization screens revealed that mutants isolated from lines NF0455, NF0549, NF0577, NF0662, NF2853, and NF3057 may contain insertions in novel common symbiotic genes, while mutants isolated from NF0342, NF0438, NF1241, and NF3037 may contain insertions in novel, specialized nodulation genes.

83.3.2 *Tnt1*-Insertion Mutant Collection as Resource for Functional Genomics in *Medicago*

Apart from the 71 Nod− mutants, the *Tnt1*-insertion symbiotic mutant collection consists of nearly 100 mutants with various defects in nodule growth and/or SNF (Fig. 83.3). These mutants were not subjected to systematic screening for insertions in known symbiotic genes, since this was beyond the scope of the original work, which was mainly to demonstrate proof of concept (Pislariu et al., 2012). Identification of disrupted genes in these, and all other mutants, relies now on the development of high-throughput sequencing strategies to efficiently recover as many FSTs as possible. Identification of candidate genes is being done by cross-referencing genes with *Tnt1* insertions with their expression profiles in the *Medicago* Gene Atlas (MtGEA) (http://mtgea.noble.org/v3/) and the generation of segregating populations to confirm cosegregation of insertions with the observed phenotypes (Fig. 83.2). If a gene for an interesting mutant is discovered, a PCR-based reverse screening is then conducted in the entire mutant population to identify additional *Tnt1*-insertion alleles. Ultimately, the positive gene identification is confirmed by the similar phenotype observed in all insertion alleles and/or by complementation. By using this strategy, we identified several novel symbiotic genes that are currently being characterized functionally (Pislariu et al., unpublished results; Sinharoy et al., unpublished results). So far, 90 FSTs in nodule-expressed genes and 11 nodule-specific FSTs have been retrieved from *Tnt1*-insertion symbiotic mutants, including Medtr6g006140.1 (putative glucose transporter); Medtr8g088740.4 (Ser/Thr protein kinase); Medtr7g013170.1 (Beta-glucan-binding protein); Medtr1g104780.1 (Hexose transporter); Medtr4g085800.1 (phosphoinositide-specific phospholipase C-like protein); Medtr6g052300.1 (cystathionine β-synthase domain-containing protein) (Sinharoy et al., unpublished results); Medtr8g018570.1 (nodule- and Myc-specific lipoxygenase); and Medtr7g009730.1 (nodule- and Myc-specific WRKY transcription factor 73) (Sinharoy et al., unpublished results). From the seven *defective in nitrogen fixation* (*dnf1-7*) mutants (Mitra and Long, 2004; Starker et al., 2006; Pislariu and Dickstein, 2007), two genes have been clones so far: *DNF1* (Wang et al., 2010) and *DNF2* (Bourcy et al., 2013). Cloning of *DNF2* was facilitated by the *Tnt1*-insertion mutant collection. Among the 51 Nod+Fix− mutants were NF0217 and NF2496, which displayed similar phenotypes, with small, white, or brownish nodules, and stunted plant growth. *Tnt1* flanking sequences corresponding to the Medtr4g085800 gene that encodes a phosphatidylinositol-specific phospholipase C X domain (PI-PLCXD)-like protein were recovered from both lines. Subsequently, it was demonstrated that these mutants (designated *dnf2-2* and *dnf2-3*, respectively), together with an additional *MERE1*-insertion mutant (*dnf2-4*), belong to the same complementation group with the Jemalong *dnf2-1* mutant. It is conceivable that more of the potentially new *Tnt1*-insertion mutants may be alleles of EMS or deletion mutants with similar phenotypes. Nearly 44,000 FSTs have so far been recovered from the entire *Tnt1*-insertion mutant population, and the goal is to have ~200,000 FSTs deposited in the mutant database by the end of 2014.

The *Medicago Tnt1*-insertion population is also a valuable resource for reverse genetics. An efficient PCR reverse-screening platform has been developed at the Noble Foundation and *Tnt1* insertions have been identified in 715 genes, representing 85% of all genes tested (Fig. 83.2) (Cheng et al., 2011). An average of 1.7 insertion alleles for each gene has been achieved. Several transcription factors and transporters, for which multiple alleles have been isolated, are currently being characterized (Sinharoy et al., unpublished results; Kryvoruchko et al., unpublished results). Furthermore, the identity of several genes cloned from EMS and FNB mutant collections (generated in the A17 background) was confirmed by isolating additional R108 alleles in the *Tnt1*-insertion mutant population (Murray et al., 2011; Horvath et al., 2011). Approximately 25 genes that, according to transcriptomic data, were predicted to play roles in symbiosis have been characterized in more or less detail in the absence of mutants (Table 83.1). Although biochemical and pharmacological experiments can provide clues about the molecular function of a protein, the exact role in symbiosis is hard to predict when corresponding mutants are not available. For example, it has been shown that root hair curling and IT formation are inhibited by the phosphatidylinositol-specific phospholipase C inhibitor U-73122 (Peleg-Grossman et al., 2007). This result, however, seems to be inconsistent with the symbiotic phenotype observed in the *dnf2* phospholipase C mutants, which develop smaller but more numerous nodules than the wild type (Bourcy et al., 2013). It is, therefore, important to isolate mutants in order to accurately assess the biological function of a gene. Some of the genes in Table 83.1 have already been targeted for PCR reverse screening to identify *Tnt1*-insertion alleles and, ultimately, gene function.

83.4 DISCUSSION

Tnt1-insertion mutagenesis has been established as an excellent tool for large-scale, saturation mutagenesis in *Medicago*. From 230 lines with putative symbiotic phenotypes, we isolated 179 mutants impaired at various stages of symbiosis. From 83 Nod− and Nod++ mutants, we found 39 insertion alleles in 9 known symbiotic genes. Several Nod−, Nod+/−Fix−, Nod+Fix−, and Nod+Fix+/− mutants have symbiotic phenotypes unlike others described in the literature, and may, therefore, be impaired in novel symbiotic genes.

The available genome sequence and transcriptomic resources for functional genomics have placed *Medicago* at the forefront of genetic studies aimed at a better understanding of SNF and AM (see Chapter 78). From transcriptomic studies, it is clear that several thousand genes are involved in these symbioses, although few of them have been characterized at the molecular level. Although members of the common symbiotic pathways and a few nodulation- and mycorrhization-specific genes have been described in recent years, there are many gaps in our knowledge of the molecular and biochemical events required to establish these mutualistic symbioses. A lot of work has been focused so far on the early steps required for the establishment of symbioses, which is indeed critical. However, molecular mechanisms occurring downstream of the rhizobium-specific (*ERN1*, *NIN*) and mycorrhizal-specific (*RAM2*) (Wang et al., 2012) genes are poorly understood at this time. It is also important to learn more about the genetic control of nodule senescence, which could have implications in the extended persistence of efficient, nitrogen-fixing nodules.

For many years RNAi has been used to infer biological function, although gene knock-down rather than knock-out is a more common occurrence, which complicates data interpretation. Nevertheless, nearly 40 *Medicago* symbiotic genes have been characterized using this method (Table 83.1). One advantage of *Tnt1*-insertion mutagenesis is that it causes mostly KO mutations, especially when insertions are exonic, toward the 5′-end of the gene or in the proximal region of the promoter.

The *Medicago* tobacco retrotransposon *Tnt1*-insertion mutant collection has also been instrumental in the cloning and characterization of *Medicago* genes not involved in symbiosis (Tadege et al., 2011; Laurie et al., 2011; Chen et al., 2010; Pang et al., 2009; Wang et al., 2008; Zhao et al., 2010; Zhou et al., 2011). As previously described, *Tnt1* transposition in *Medicago* offers advantages that have not been surpassed by other mutagenesis methods in the past 6 years (d'Erfurth et al., 2003; Tadege et al., 2005). They include transposition to unlinked sites, multiple insertions per line that should allow near-complete genome coverage; insertion primarily within coding sequences; generation of mostly KO mutations; stability during seed-to-seed propagation (copy-and-paste transposition); and simplification of gene cloning by the ease of FST recovery. There are some drawbacks, such as the tissue culture step that is required for the *Tnt1* activation, with the potential of triggering endogenous mobile elements, and somaclonal variation (Larkin and Scowcroft, 1981). Also, having multiple insertions in other genes can complicate genetic analysis and necessitates the use of at least two alleles and/or backcrosses for cosegregation analysis. This contrasts with the *Arabidopsis* T-DNA lines, which average 1.5 insertions per line. However, *Medicago* is larger and slower to set seed than *Arabidopsis*, and has a much larger genome, so generation of a saturation population using T-DNA would be prohibitively expensive, while using *Tnt1* has allowed this goal to be achieved using a much smaller population size and in shorter time.

The robustness of *Tnt1*-insertion mutagenesis has been confirmed by the large number of publications reporting gene cloning and functional characterization using this resource, and by the attempt to extend the same technology to soybean (Cui et al., 2013). The *Medicago Tnt1*-insertion population is an excellent tool for gene discovery and molecular characterization using both forward and reverse genetic approaches. Improving FST recovery will certainly speed up the identification of novel key symbiotic genes. Furthermore, conducting large-scale PCR reverse screenings for *Tnt1* insertion alleles of genes of interest will help clarify the biological functions of partially characterized genes for which no mutants were available previously.

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Section 15

Cyanobacteria and Archaea

Chapter 84

Marine Nitrogen Fixation: Organisms, Significance, Enigmas, and Future Directions

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84.1 INTRODUCTION

The biological productivity of the oceans is constrained by the cycling and biological availability of nutrients, of which nitrogen (N) is critical (Gruber and Galloway, 2008; Canfield et al., 2010). N is primarily recycled in the open ocean through remineralization of organic matter. A primary new N input comes from upwelling of deep water containing nitrate formed from remineralization and oxidation of the organic matter, which sinks through the water column. However, other important inputs of N are atmospheric deposition of fixed N compounds, terrestrial runoff, and biological N_2 fixation (Seitzinger and Kroeze, 1998; Jickells, 2006; Duce et al., 2008; Gruber, 2008). Some marine biological N_2 fixation occurs in relatively small, localized habitats such as seagrass beds and sediment microbial mats, where N input is important ecologically for that specific habitat or ecosystem (Capone, 1983, 1988; Bebout et al., 1994). However, biological N_2 fixation also occurs in the vast expanses of the open ocean and is globally the largest marine input, and it balances N losses from the mixed layer to the deep sea and sediments, as well as losses to the atmospheric N_2 pool via denitrification and anaerobic ammonia oxidation.

In the past two decades, continued new discoveries have been made about the organisms involved, how N_2 fixation is distributed in the oceans, and the factors that control N_2 fixation in the sea (Ward et al., 2007).

84.2 N_2 FIXATION, MARINE HABITATS, AND CYANOBACTERIA

Biological N_2 fixation is catalyzed by diverse bacteria and Archaea, but not by Eukaryotes, although some bacteria and Archaea fix N_2 in symbiosis with eukaryotes (see below). In the marine environment, there are many habitats that contain diverse N_2-fixing microorganisms (diazotrophs) (Fig. 84.1) from all of these groups (Table 84.1). Open ocean N_2-fixing habitats range in temperature and pressure and include warm tropical and subtropical surface waters, and also cold polar and cold mesopelagic- and deep ocean waters. Benthic habitats include sediments, coral reefs, and benthic microbial mats (Zehr and Paerl, 2008). Marine habitats also include special environments such as hydrothermal vents, characterized by high temperatures and are rich sources of electron donors.

The N_2-fixing microbial taxa generally differ among these marine habitats (Table 84.1) and include Archaea and diverse (photo)heterotrophic and chemolithotrophic bacteria and photoautotrophic cyanobacteria. For example, archaeal nitrogenase (*nifH*) genes have been found in deep water and near hydrothermal vents (Mehta et al., 2003; Mehta and Baross, 2006; Dekas et al., 2009). Many heterotrophic bacterial *nifH* genes are typically found in sediments (Capone, 1983; Dang et al., 2009), salt marshes (Moisander et al., 2005), and microbial mats (Olson et al., 1999;

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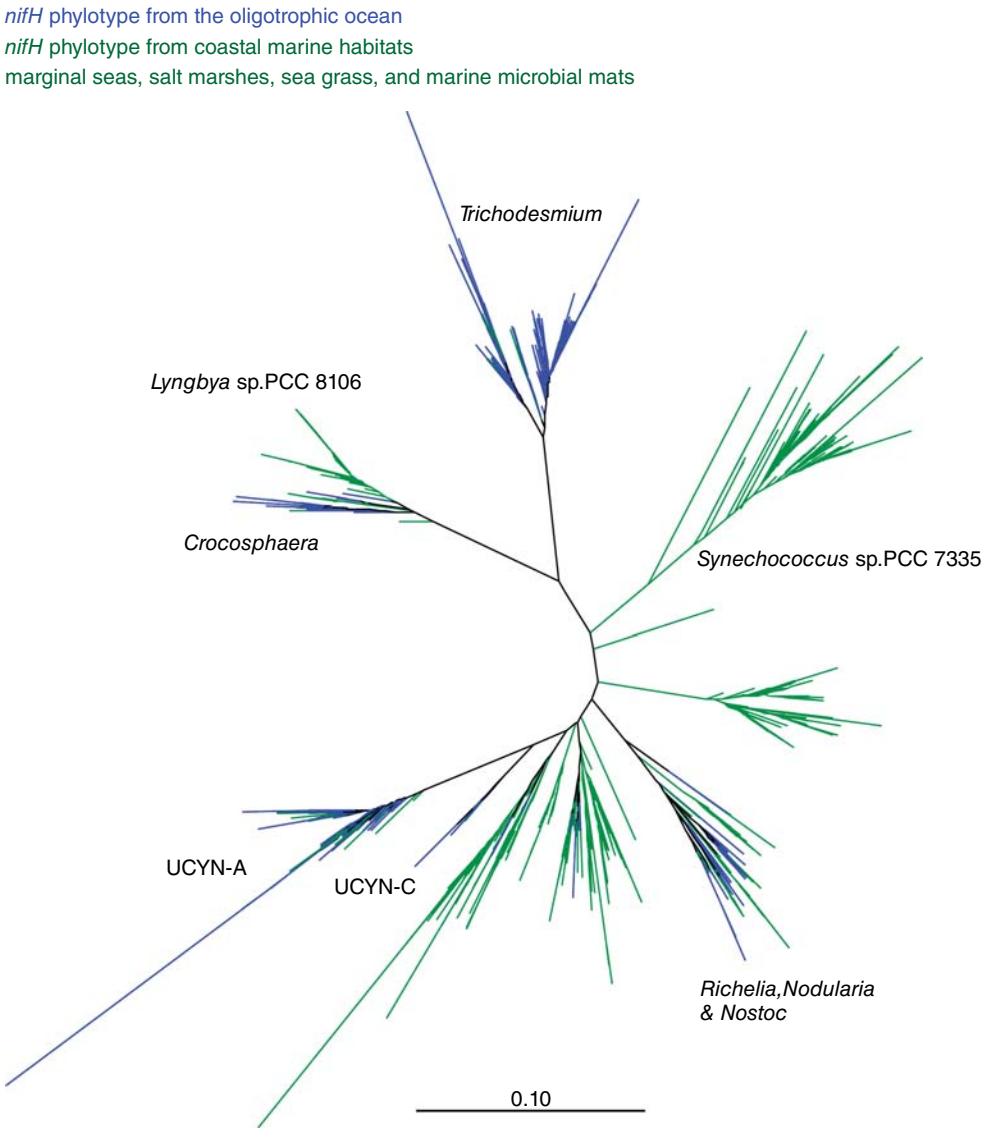

Figure 84.1 Phylogenetic distribution of cyanobacterial (*nifH* cluster 1B) *nifH* genes in the marine environment.

Moisander et al., 2006; Severin and Stal, 2010) (Table 84.1). Thus, marine diazotrophs can thrive across large ranges in temperature, salinity, and oxygen, and these variables themselves do not constrain N_2 fixation in the marine environment.

Cyanobacteria are obvious and important components of many of these marine habitats, such as in microbial mats (Zehr and Paerl, 2008; Paerl, 2012). Cyanobacteria of a variety of morphologies and phylogenies are involved in N_2 fixation in microbial mats, ranging from filamentous heterocyst-forming and nonheterocyst-forming species to unicellular N_2-fixing species (Zehr and Paerl, 2008; see Chapters 85, 86). Strikingly, the N_2-fixing cyanobacteria in the ocean water column are represented by only a handful of lineages, in contrast to the rich diversity of microbial mats (Zehr and Paerl, 2008) (Fig. 84.1).

Heterotrophic bacteria in the ocean water column have largely been ignored until recent reports of heterotrophic bacterial *nifH* gene expression (Zehr et al., 2001; Bird et al., 2005; Church et al., 2005b; Bird and Wyman, 2012) and high *nifH* sequence diversity (Riemann et al., 2010; Farnelid et al., 2011) in the open ocean (Fig. 84.2). This review focuses primarily on what is known about N_2-fixing cyanobacteria in the open ocean, but will also touch upon aspects of the potential for heterotrophic or (photo)heterotrophic N_2 fixation.

Table 84.1 Representatives of Major Groups of N_2-Fixing Microorganisms in Marine Habitats

Environment	*nifH* Cluster	Phylym/Subphylum	Strain/Phylotype	References (examples)
Marine Oligotrophic Euphotic	Cluster I	Cyanobacteria	*Crocosphaera*, *Cyanothece*-like, *Katagnymene*-like, *Richelia* in *Rhizosolenia*, *Richelia*-like, *Trichodesmium*, UCYN-A	Zehr et al., 1998, Langlois et al., 2005
		α-proteobacteria	24809A06, Bradyrhizobium-like, Mesorhizobium, Rhodobacteraceae	Falcon et al., 2004, Turk et al., 2011
		β-proteobacteria	*Burkholderia*-like, uncultivated phylotype(s)	Zhang et al., 2011
		γ-proteobacteria	24774A11, *Klebsiella*-like, uncultivated phylotype(s)	Moisander et al., 2008
	Cluster III	δ-proteobacteria	*Dehalococcoides*-like, *Desulfovibrio*-like	Farnelid et al., 2011
Marine Coastal	Cluster I	Cyanobacteria	*Lyngbya*-like, Nostocales-like, *Trichodesmium*-like, UCYN-A	Bauer et al., 2008, Messer et al., 2015
		α-proteobacteria	*Bradyrhizobium*-like, *Burkholderia*-like, Rhodobacteraceae, uncultivated phylotype(s)	Fernandez et al., 2011, Kong et al., 2011
		β-proteobacteria	*Azoarcus*-like, uncultivated phylotype(s)	
		γ-proteobacteria	*Marichromatium*-like, uncultivated phylotype(s), *Chloroflexi*-like, uncultivated phylotype(s)	
	Cluster III	δ-proteobacteria	*Desulfovibrio*-like	Bombar et al., 2011
		Firmicutes	*Clostridium*-like	Bentzon-Tilia et al., 2014
		Other cluster III	other uncultivated phylotype(s)	

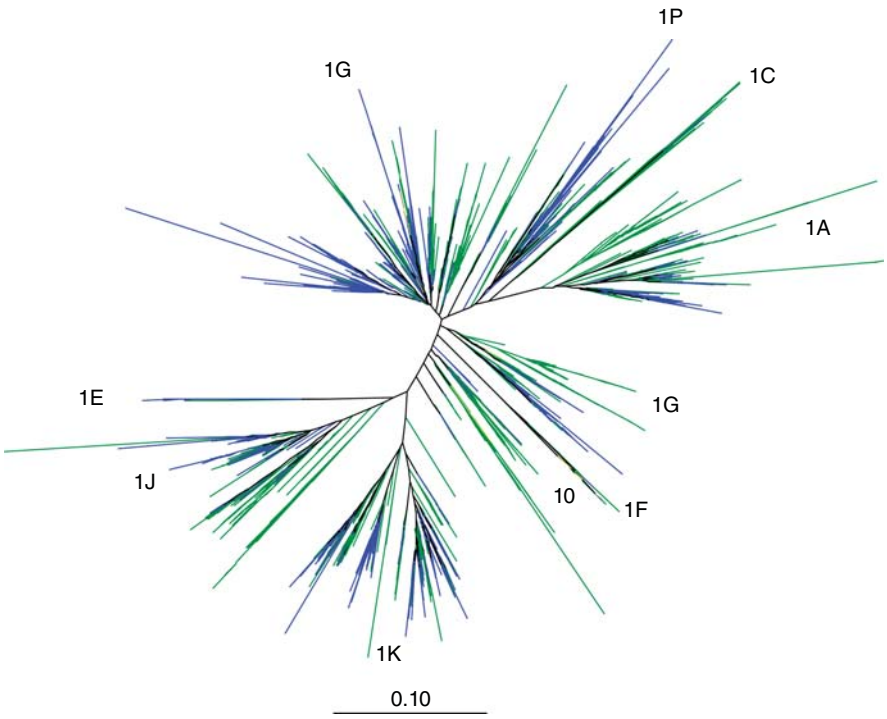

Figure 84.2 Phylogenetic distribution of cluster 1 *nifH* genes (bacterial) in the marine environment.

84.3 WHY IS N_2 FIXATION IN THE OPEN OCEAN INTERESTING?

On land and in the sea, the availability of nutrients constrains the productivity of the biosphere (Vitousek and Howarth, 1991). In the oceans, the primary nutrients and trace elements that limit growth and productivity are N, phosphorus (P), and iron (Fe). The water-column microbes of the vast open sea dominate the C and N fluxes of the ocean (Longhurst, 1991; Raven and Falkowski, 1999; Zehr and Kudela, 2011). Primary productivity in the surface waters of the oceans is roughly equivalent to that on land (Field et al., 1998), and is mainly carried out by the unicellular cyanobacteria *Synechococcus* and *Prochlorococcus* (Johnson and Sieburth, 1979; Waterbury et al., 1979; Chisholm et al., 1988). Similarly, N_2 fixation in the water column of the ocean dominates global ocean N_2 fixation (Fowler et al., 2013; Voss et al., 2013), and is roughly equivalent to the magnitude of anthropogenic N_2 fixation in agriculture and industry (Fowler et al., 2013). In the oceans, marine biological N_2 fixation, along with upward fluxes of deep water NO_3^- to the surface ocean, fuels primary productivity and quantitatively balances the losses by sinking of organic material, which sequesters CO_2 from the atmosphere to deep waters (Karl et al., 1997; Sohm et al., 2011).

Although N limits the productivity and the ability of the oceans to sequester CO_2, equally, or ultimately more important, is the availability of other nutrients that control the growth of photosynthetic- and N_2-fixing microorganisms (Mills and Arrigo, 2010; Sohm et al., 2011). Phosphorus (P), which does not have a significant reservoir as a gas in the atmosphere, is largely sequestered in rocks and sediments (Delaney, 1998), and can be an important limiting factor for diazotrophs and primary producers in the sea (Wu et al., 2000; Karl et al., 2001; Sanudo-Wilhelmy et al., 2001; Mills et al., 2004; Hynes et al., 2009; Kitajima et al., 2009). The trace element Fe, important for many enzymes (including but not limited to nitrogenase), is often in limiting supply because it is insoluble in oxidized seawater and also plays a critical role in the environmental control of photosynthetic CO_2 fixation and N_2 fixation (Webb et al., 2001; Wu et al., 2001, 2003; Boyd et al., 2007; Moore et al., 2009; Chappell et al., 2012; Shi et al., 2012). In the open ocean, the factors that control the availability of these nutrients and trace elements include large-scale processes, such as global and local water mass circulation (Palter et al., 2011), and atmospheric wind transport and deposition of dust (Baker et al., 2003; Bonnet and Guieu, 2004; Mills et al., 2004; Duggen et al., 2010). Due to the vast expanse of the oceans (Fig. 84.3) and the difficulties of sampling the ocean over the necessary large scales, gaining global-level estimates of N_2 fixation and the organisms responsible is extremely challenging (Church et al., 2009; Voss et al., 2013; see Chapter 87).

84.4 N_2 FIXATION IN THE SEA

For many years, rates of N_2 fixation in the open sea were assumed to be negligible, or at least biogeochemically unimportant (Mague et al., 1974; Carpenter and McCarthy, 1975; Eppley and Peterson, 1979). This assumption changed when it was demonstrated that *Trichodesmium*, a filamentous cyanobacterium that is easily observed because of its tendency to form aggregates large enough to see with the unaided eye, could fix N_2 (Dugdale et al., 1961). In the ensuing years, pioneering N_2 fixation work showed that *Trichodesmium* was widely distributed and that N_2 fixation occurred in many places in the surface ocean, but was highly variable temporally and spatially (Dugdale et al., 1964; Carpenter, 1973, 1983; Mague et al., 1974; 1977; Carpenter and Culliney, 1975; Carpenter and Price, 1977; Saino and Hattori, 1978; Capone et al., 1997).

Interest and research focus on N_2 fixation intensified in the late 1990s because biogeochemical calculations predicted that there must be much greater rates of oceanic N_2 fixation than previously estimated (e.g., from *Trichodesmium* rates and abundances) (see Chapter 87). This interest in oceanic N_2 fixation led to new discoveries about the organisms and processes responsible for controlling open ocean N_2 fixation (Ward et al., 2007; Zehr, 2011)

84.5 WHAT ARE THE N_2 FIXATION RATES OF THE OCEANS?

Determining reliable rates of N_2 fixation, a seemingly simple experimental measurement, is in reality a very challenging endeavor. Since sampling for N_2 fixation rates even within a single ocean basin is time- and money-intensive, some of the best large-scale estimates are not based on direct experimental rate measurements, but rather on mathematical models (Monteiro et al., 2010, 2011) or biogeochemical estimates (Gruber, 2005; Deutsch et al., 2007). There is no accurate way to assess N_2 fixation in the ocean by remote sensing, as has been used for primary productivity (Behrenfeld and Falkowski, 1997), except maybe in very special situations, such as surface blooms of *Trichodesmium*, which can cover hundreds of miles and are visible on satellite images (Capone et al., 1997; Subramaniam et al., 1999; Westberry and Siegel, 2006). However, satellite remote sensing does not detect subsurface biomass, and biomass cannot be easily translated to N_2 fixation rates. Biogeochemical models based on regenerated nutrient stoichiometry (N and P) can be used to predict basin-scale rates and regional patterns of N_2 fixation (and denitrification). It was these types of estimates that led to the conclusion that oceanic N_2 fixation rates were higher than previously assumed (Michaels et al., 1996; Gruber and Sarmiento, 1997; Lee et al., 2002; Codispoti, 2007). These models are based on a variety of assumptions (stoichiometry

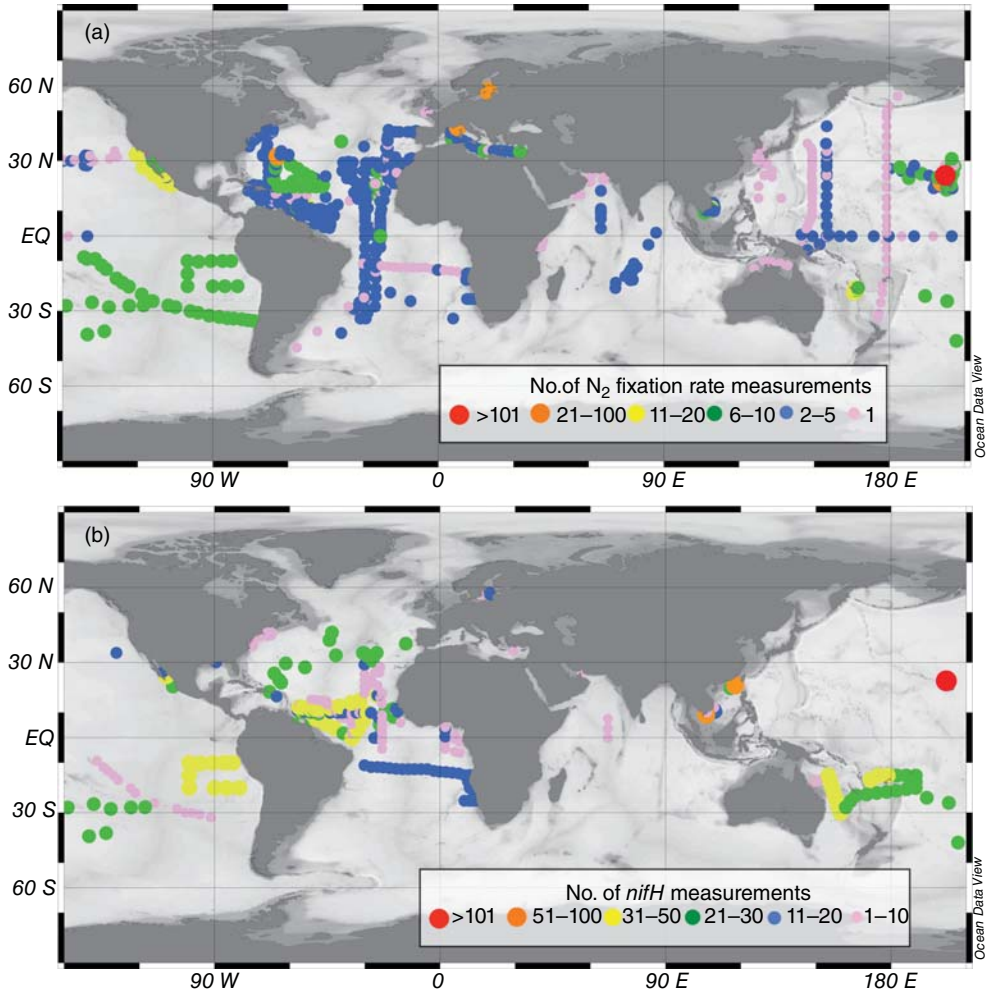

Figure 84.3 Global map of N_2 fixation rate measurements and quantitative *nifH* assays (modified after Luo et al., 2012).

of nutrients in microorganisms, rates of sedimentation, water mass flow rates, scales of integration), and thus the calculated magnitude of global N_2 fixation (in the range of $100–200 \times 10^{12}$ g N/year) strongly varies between different studies (Gruber and Sarmiento, 1997; Hansell et al., 2004; Koeve and Kahler, 2010), and the first higher estimates of N_2 fixation rates more closely balanced known oceanic denitrification rates (Hansell et al., 2004; Gruber, 2005; Codispoti, 2007).

It would seem that the most direct way to determine N_2 fixation rates is to measure rates using conventional stable isotope ($^{15}N_2$) or acetylene reduction methods. This is not as simple as it may seem to the nonoceanographer because of the large horizontal and vertical scales of variability, and because of the dilute nature of the oligotrophic (low N) environment. For many years, oceanic N_2 fixation rates were estimated by incubating hand-picked macroscopic colonies of *Trichodesmium*, but the discovery of microscopic unicellular N_2 fixers (see later) led to the need for bulk seawater measurements. However, since N_2-fixing microorganisms are not very abundant relative to dominant species, measuring rates of $^{15}N_2$ incorporation (or acetylene reduction) in bulk seawater are difficult due to the limits of detection. Recently, there have also been suggestions of experimental artifacts using the traditional $^{15}N_2$ gas bubble addition assay in seawater (Mohr et al., 2010), which may differentially bias rate measurements from different types of microorganisms (Grosskopf et al., 2012). In addition, considering the size and variability of the ocean ecosystem, it appears impossible to use shipboard sampling (which is constrained in space and time) to carry out representative incubations that cover the range of seasonality, and small- and large-scale patchiness of microorganisms and nutrients over the vast ocean basins. Depending on the spatial resolution achieved during different research cruises, estimates regarding the magnitude of N_2 fixation in a particular area can vary considerably (Shiozaki et al., 2009, 2010; see Chapter 87).

Another approach to estimate oceanic N_2 fixation rates is to identify the responsible organisms, to determine their distributions and activity, and to reveal the factors that control their distributions and activity. This information is then used to model rates (Goebel et al., 2007, 2010) or the global patterns of N_2 fixation (Monteiro et al., 2010). This approach requires obtaining data on the abundance of the organisms, their specific N_2 fixation rates in seawater (or good estimates of cell-specific rates of N_2 fixation, (Goebel et al., 2010)) and on the nutrients that control the distribution and activities of these organisms in order to develop models related to the fluxes of these nutrients (P and Fe). It is not easy to identify N_2-fixing microbes in a drop of seawater, let alone to measure their specific rates of N_2 fixation. *Trichodesmium* is easily observed and concentrated by net tows. Thus, even though typical high concentrations of *Trichodesmium* are only a few cells per milliliter of seawater, enough *Trichodesmium* can be collected from long net-tows to obtain a reliable rate, which can be extrapolated over large volumes of seawater. This is generally true of the diatom symbionts as well. However, the unicellular cyanobacteria, including the UCYN-A symbiont, are extremely difficult to detect, and it is difficult to measure rates from a few cells per milliliter of small organisms that cannot be visualized or easily concentrated to make similar rate measurements. Also, some of these organisms can only be quantified by molecular techniques, which means that the organisms are not detected until long after sampling. Rates determined in bulk water when multiple species are present make it difficult to deconvolute individual species rates (Goebel et al., 2010). The use of stable isotopes and nanoscale secondary ion mass spectrometry (nanoSIMS) has made it feasible to determine N_2 fixation rates on individual cells (Finzi-Hart et al., 2009; Foster et al., 2011, 2013), but the variability among cells and incubation conditions challenge scaling up to large areas. Attempts to model the relative contributions of different N_2 fixers rely on limited available microscopic counts and the abundance data from quantitative PCR (Luo et al., 2012) (Fig. 84.2). Thus, models need to extrapolate in space and time from a relatively small data set, considering the size of the oceans, and they need to consider the seasonality of plankton communities.

The ability to estimate or model integrated N_2 fixation from measured rates and/or organismal abundances is constrained by the frequency and spatial coverage of sampling by ships or with remote instruments (Fig. 84.3). Recent development of a N_2 fixation database provides the first comprehensive amalgam of such information from diverse sources (Luo et al., 2012) and also highlights the problem of uneven spatial coverage of N_2 fixation studies (Fig. 84.2) and diverse sampling procedures (Zehr and Kudela, 2011) associated with different surveys. The expense and logistics of oceanographic cruises often led to research being carried out in areas where N_2 fixation was known to occur, such as the Atlantic Ocean and Caribbean Sea or the North Pacific (Fig. 84.3). In recent years, a number of longer survey transects have been sampled, although methods and measurements differ among studies, making it difficult to define the regions, temporal dynamics, and rates of N_2 fixation in the global ocean (Zehr and Kudela, 2011). However, such surveys have led to hypotheses regarding the general patterns of N_2 fixation in the oceans (Sohm et al., 2011).

Models and extrapolations are also sensitive to the unknown physiological status at the time of sampling and the general controlling factors in the ocean. The now known symbiotic relationship of UCYN-A represents a further complication, since it is unknown how cell-specific rates of UCYN-A vary (Goebel et al., 2010) and how they are regulated within the symbiosis with a photoautotrophic eukaryote. Thus, the question of whether *Trichodesmium* dominates oceanic N_2 fixation rates is not resolved and will not be easily resolved. However, the much greater geographic distribution of UCYN-A compared to *Trichodesmium* suggests that the integrated global rates will be greatly affected by UCYN-A. Recently, instrumentation has been developed that can perform quantitative PCR *in situ*, and deployment of that enables unmanned detection of N_2-fixing microorganisms (Robidart et al., 2014). Ultimately, this type of instrumentation may help to obtain higher resolution data on the abundances of different diazotrophs than has been acquired by oceanographic expeditions (Fig. 84.3).

84.6 OCEANIC N_2-FIXING MICROORGANISMS AND CHANGES IN PARADIGMS

Studies of N_2-fixing microorganisms in the oceans based on molecular biology and genomics have led to the revision of a number of dogmas about marine N_2 fixation. Following are several examples of previous dogmas, and how recent findings have led to important changes in what we know about N_2 fixation.

1. *Trichodesmium*, a filamentous, nonheterocyst-forming cyanobacterium, was believed to be the only important N_2 fixer in the oceans (Capone et al., 1997; LaRoche and Breitbarth, 2005). This assumption led to a variety of conclusions and development of mathematical models (Coles et al., 2004; Hood et al., 2004) that predicted where and when N_2 fixation occurs.

   It is now known that *Trichodesmium* is not the only important N_2-fixing cyanobacterium in the sea. Early microscopic studies showed that there were other cyanobacteria, especially the heterocyst-forming symbionts of diatoms, present in ocean waters (Carpenter and Capone, 1983; Villareal and Carpenter, 1989). More recently, applications of molecular biology and genomic approaches uncovered unexpected new microorganisms

involved in oceanic N_2 fixation. Although it may be that *Trichodesmium* still dominates oceanic N_2 fixation, it is necessary but difficult to assess the relative contributions of the less easily observed and uncultured unicellular cyanobacteria (Zehr et al., 2000; Capone, 2001).

2. Oceanic N_2 fixation is constrained to warm surface waters. This dogma arose primarily because of the belief that *Trichodesmium* is the primary N_2 fixer, and it is largely only found in warm tropical or subtropical waters.
   The discovery that other N_2-fixing cyanobacteria have different temperature optima (Needoba et al., 2007; Langlois et al., 2008; Moisander et al., 2010) and are more widely distributed geographically, including at high latitudes (Rees et al., 2009; Blais et al., 2012; Diez et al., 2012) and in coastal waters (Mulholland et al., 2012), has challenged this dogma. The integrated rates over the greater latitudinal range may change global estimates of oceanic N_2 fixation.
3. N_2 fixation does not occur in waters with fixed inorganic N. N_2-fixing microorganisms are believed to be only competitive when fixed inorganic N is not available, based on a relatively small number of studies of ecological competition between N_2-fixing and non-N_2-fixing microorganisms. Also, since N_2 fixation is an energetically expensive process, it is assumed to be highly regulated by the cell (in response to oxygen and fixed N availability) and not expressed when fixed inorganic N is present (Mulholland and Capone, 2000; Holl and Montoya, 2005; Knapp, 2012). There have been many studies of model microorganisms that demonstrate the regulation of N_2 fixation genes and N_2 fixation activity in response to the presence of fixed inorganic N. These assumptions led to the focus of sampling and research on N_2 fixation in oligotrophic waters with extremely low fixed-N concentrations (Capone et al., 1997) and to the conclusion that N_2 fixation does not occur in coastal or upwelling waters, or in sediments, where fixed inorganic N concentrations are relatively high.
   The finding of N_2 fixation in nutrient-enriched upwelling regions appears to contradict these assumptions. Also, the studies on which this dogma is founded involve experiments with relatively high concentrations of fixed N (e.g., millimolar ammonium or nitrate), and other studies have shown that some N_2-fixing cyanobacteria will fix N_2 in the presence of fixed N (Ohki et al., 1991; Mulholland et al., 1999, 2001; Holl and Montoya, 2005; Knapp, 2012). Furthermore, N_2 fixation has been demonstrated to be important in upwelling areas or near riverine inputs where fixed N is present, albeit at low concentrations (Voss et al., 2006; Foster et al., 2007; Subramaniam et al., 2008; Bombar et al., 2011). N_2 fixation is only slightly more energetically expensive than using NO_3^- (Karl et al., 2002), making the argument that N_2 fixers are not competitive when fixed inorganic N is present more complicated. Finally, N_2 fixation has been detected in sediments (Slater and Capone, 1984; Burns et al., 2002; Fulweiler et al., 2007), even in the presence of high concentrations of ammonium, suggesting that N_2 fixation proceeds even when fixed N is present in high concentrations and may be regulated or employed for a metabolic reason other than obtaining N. *Rhodobacter* in culture was shown to regulate nitrogenase expression as a function of redox conditions (Tichi and Tabita, 2000), for example. There is no direct evidence, however, that nitrogenase in the environment is expressed for any other reason than obtaining N.
4. N_2 fixation is a strictly anaerobic process. The fact that the enzyme is sensitive to inactivation by oxygen, and that model microorganisms have physiological or cellular adaptations for fixing N_2 in photosynthetic organisms or aerobic environments (Stal and Krumbein, 1985; Milligan et al., 2007; Staal et al., 2007), has led to assumptions about how and where N_2 fixation can occur and the kinds of organisms that could be capable of N_2 fixation (see Chapter 86). This assumption was one of the reasons that it was not believed that *Trichodesmium* was a N_2-fixing cyanobacterium in early studies, because it did not make heterocysts to allow simultaneous N_2 fixation and photosynthesis. Ultimately, it was clearly demonstrated that *Trichodesmium* does fix N_2, and only in the light (Dugdale et al., 1961). Many studies have attempted to determine how *Trichodesmium* can evolve O_2 while simultaneously fixing N_2 (Paerl and Bebout, 1988; Carpenter et al., 1990; Kana, 1993; Berman-Frank et al., 2003; Küpper et al., 2004), but it is a difficult problem because of its filamentous growth morphology, is somewhat controversial, and is not completely resolved (see Bergman (1999) and Thompson and Zehr (2013)).
   This assumption also questioned the significance of recently discovered unicellular cyanobacteria "UCYN-A," which appeared to fix N_2 during the light and express *nifH* genes during the light (Church et al., 2005b). Ultimately, it was found that this organism is not photosynthetic and neither evolves O_2 nor fixes CO_2 (Zehr et al., 2008; Tripp et al., 2010). However, it still lives on or in a photosynthetic unicellular alga, which is evolving O_2. In photosynthetic unicellular diazotrophs like *Cyanothece*, respiration plays an important role in quenching away oxygen to enable the cells to carry out N_2 fixation (Bandyopadhyay et al., 2013).
   More recently, it has been suggested that heterotrophic bacteria are more abundant than cyanobacteria (based on representation in amplified gene sequence libraries, such as pyrosequencing) and may also be important in

oceanic N_2 fixation (Riemann et al., 2010; Farnelid et al., 2011). It is unclear how these heterotrophic bacteria would avoid inhibition by O_2 at saturating concentrations in surface waters, but it might be plausible that they have protective mechanisms such as the high respiration rates and other mechanisms, for example, found in terrestrial *Azotobacter* (Dalton and Postgate, 1969; Oelze, 2000). It is also unclear how they would support the energetic needs of a high respiration rate, let alone the costs of N_2 fixation, using the low concentrations of dissolved organic matter in the surface ocean.

84.6.1 New Discoveries about Oceanic N_2-Fixing Cyanobacteria

N_2-fixing microorganisms, traditionally observed by microscopy, became the target for molecular biology in the late 1980s (Zehr and McReynolds, 1989; Zehr et al., 1995). Sequencing of amplified *nifH* genes was first used to confirm that *Trichodesmium* fixed N_2, and that measured N_2 fixation rates were not due to the associated heterotrophic microorganisms (Zehr and McReynolds, 1989). The molecular approach proved to be a powerful technique for characterizing diversity (Zehr et al., 1995) but, more importantly, for identifying novel microorganisms in the open ocean (Zehr et al., 1998, 2001). These approaches yielded new information on the previously known N_2-fixing cyanobacteria *Trichodesmium* and the diatom symbionts (*Richelia*), and also identified new and novel important unicellular N_2-fixing species.

84.6.2 Trichodesmium

Trichodesmium is enigmatic in that it does not form heterocysts and yet fixes N_2 only in the light (Capone et al., 1997). This enigma has been long-studied, starting with demonstrating that the aggregates fixed N_2 (Dugdale et al., 1961), that it has cyanobacterial *nifH* genes (Zehr and McReynolds, 1989), and that the nitrogenase protein is found in *Trichodesmium* cells (reviewed in Bergman et al. (2013)). Physiological and immunological evidence suggests that there is spatial variability in cell nitrogenase distribution and possibly activity, and that there are short temporal variations in photosystem II activity that could help to reduce the damaging effects of O_2 generated in photosynthesis (Bergman, 1999; Berman-Frank et al., 2001; Küpper et al., 2004). However, it still has been impossible to assay nitrogenase activity and photosynthetic activity in the same cells at the same time, and thus there is still some uncertainty in how N_2 fixation works in *Trichodesmium*. Part of the explanation might be a temporal segregation of CO_2 fixation (maximal in the morning) and N_2 fixation (peaking in the afternoon) (Finzi-Hart et al., 2009). This topic has been reviewed elsewhere (Stal and Zehr, 2008; Bergman et al., 2013; Thompson and Zehr, 2013).

84.6.3 Diatom Symbionts

Among the various examples of marine symbioses that involve diazotrophs (Fiore et al., 2010), the cyanobacterial symbionts of diatoms are an important open-ocean example. Molecular approaches showed that these symbionts, previously observed by microscopy, were composed of several distinct lineages that may be host-specific (Janson et al., 1999; Foster and Zehr 2006; Hilton et al., 2013). These cyanobacteria range in how they are physically associated with their hosts from epibiotic on the spines of *Chaetoceros* diatoms (*Calothrix*) to inside the frustule of *Hemiaulus* and *Rhizosolenia* (*Richelia*). Recently genomic sequencing of isolated filaments of *Calothrix* cultures (Foster et al., 2010) and *Richelia* isolated from *Hemiaulus hauckii* and *Hemiaulus membranaceus* showed that the genomes are substantially different, with the genomes of the endosymbiotic forms highly streamlined (Hilton et al., 2013) (Table 84.2). The genome of *Richelia* associated with *H. hauckii* is only

Table 84.2 Genome Comparison Between the Cyanobacterial Diatom Symbionts, *Calothrix* and *Richelia* (taken from Hilton et al., 2013)

	Richelia (Hh)	*Calothrix* SC01
Genome size, Mb	3.2	6.0
Transporters	55	144
GOGAT	X	✓
GS-inactivating factor	X	X
Glutaminase	X	✓
NH_4^+ transporter	X	✓
NO_3^- transporter	X	✓
Urea transporter	X	X
NO_3^- reductase	X	✓
NO_2^- reductase	X	✓
Urease	X	X
Nif insertion element	*nifH* (9.1 kb)	*nifH* & *nifK*

Calothrix is an epibiont on the diatom *Chaetoceros*, and *Richelia* is an endosymbiont in diatom *Hemiaulus* spp.

3.2 Mb, whereas that of *Calothrix* is 6.0 Mb. More interestingly, the *H. hauckii* endosymbiont is lacking many core N metabolism genes, including ammonium transporters and glutamine oxoglutarate aminotransferase (GOGAT). Clearly, genome streamlining appears to be correlated with the extent of the symbiotic association.

84.6.4 Unicellular Cyanobacteria (UCYN-A, B, and C)

NifH gene sequences that were phylogenetically closely related to unicellular strains were the first clues that *Trichodesmium* was not the only N_2-fixing cyanobacterium in the open ocean. Some *nifH* gene sequences amplified from oceanic water samples were phylogenetically related to the *Cyanothece* clade. There have been three groups of sequences found, called Groups A, B, and C (now called UCYN-A, B, and C (unicellular cyanobacteria)) (Langlois et al., 2005; Foster et al., 2007). Group B sequences were virtually identical to the sequences from *Crocosphaera watsonii*, which had been cultivated from the Atlantic (Zehr et al., 2001, 2007), but had not previously been recognized as being a widely distributed important ocean N_2-fixing cyanobacteria. *Crocosphaera*-like cyanobacteria had been observed by microscopy or flow cytometry a few times (Campbell et al., 1983; Neveux et al., 1999), but molecular approaches first documented how widely spread and common they are (Mazard et al., 2004; Church et al., 2005b; Hewson et al., 2009; Webb et al., 2009). A representative of UCYN-C (Foster et al., 2007), which appears to be not as common as UCYN-A or -B, has recently been cultivated from the oligotrophic western Pacific Ocean (Taniuchi et al., 2012).

UCYN-A was known only from its *nifH* gene for many years, as it could not be cultivated or identified microscopically. By coupling flow cytometry with high-throughput sequencing, the genome sequence was obtained (Zehr et al., 2008) and was closed (Tripp et al., 2010). The surprising finding was that although evolutionarily closely related to the *Cyanothece* clade, the genome was only 1.44 Mb and lacked many metabolic pathways, including those usually assumed to define cyanobacteria, like the oxygen-evolving photosystem II and Rubisco. This clearly suggested that the "cyanobacterium" was a symbiont. By coupling flow cytometric sorting (Zehr et al., 2008) with *in situ* hybridization to identify UCYN-A (Krupke et al., 2013), and nanoSIMS to visualize $^{15}N_2$ and $^{13}CO_3$ fixation, a symbiotic eukaryotic

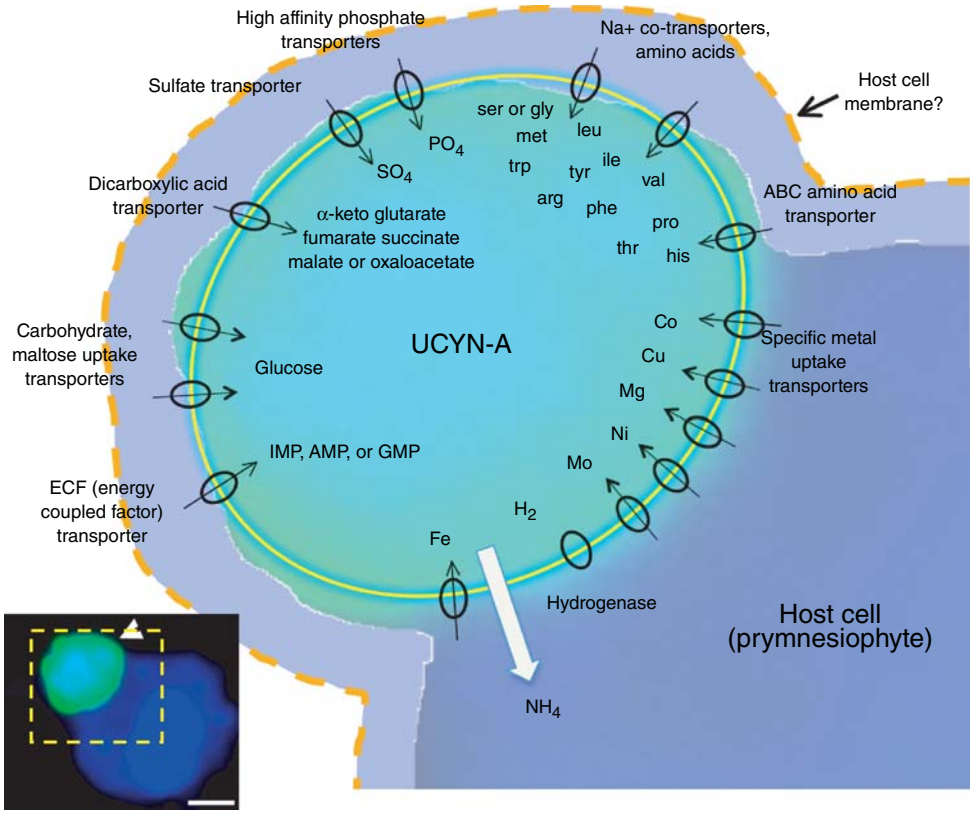

Figure 84.4 Proposed basic metabolism of the unicellular symbiotic cyanobacterium *Candidatus* Atelocyanobacterium thalassa (UCYN-A). The scale bar represents 1 μm.

prymnesiophyte partner was identified (Thompson et al., 2012). The host, or partner, was the first known example of a symbiosis with a haptophyte unicellular algae. UCYN-A cannot fix CO_2 and lacks the ability to synthesize several amino acids and purines (Tripp et al., 2010). The genome sequence of UCYN-A provides clues as to how metabolites such as sugars, dicarboxylic acids, amino acids, and other nutrients are exchanged between the host and symbiont (Fig. 84.4). Sugars are presumably degraded via glycolysis to pyruvate, and further to acetyl CoA and CO_2, thereby providing energy. The lack of ability for biosynthesis of basic cellular building blocks and energy substrates, and overall the extensive degree of genome streamlining, suggest that this symbiosis is obligate. However, it remains unclear whether UCYN-A is an endosymbiont, and how cells are vertically transmitted by the dividing host cells. It is known that the association is relatively fragile (Thompson et al., 2012), and UCYN-A has at times presumably been observed as free cells (Krupke et al., 2013). Thus, there are important details about this symbiosis yet to be revealed. Furthermore, there may be more symbioses yet to be discovered. UCYN-A was only discovered because of the focus of research to elucidate the organism associated with the UCYN-A *nifH* gene sequence. Other cyanobacteria have been observed in association with eukaryotic algae, including diatoms and dinoflagellates (Carpenter and Foster, 2002; Janson, 2003; Foster and O'Mullan, 2008). Also, there are possibly other types of interactions involving diazotrophy, such as for the marine diazotroph cyanobacteria *Leptolyngbya nodulosa*, which only express functional nitrogenase when growing together with heterotrophic bacteria (Li et al., 2010).

84.7 OTHER KNOWLEDGE GAPS

84.7.1 Different Ecosystem Roles of Cyanobacterial Diazotrophs

It is likely that the major oceanic diazotroph species have been identified (although the potential of heterotrophs remains unresolved), but we are just beginning to understand the complex ecological roles of each type in an ecosystem context. From a biogeochemical perspective, the obvious role of these diazotrophs is to provide new N to the ocean, but the different types of diazotrophs have different effects and fates. Depending on their lifestyle, some diazotrophs also photosynthesize (such as *Trichodesmium*, *Crocosphaera*) or provide N to photosynthesizing symbiotic hosts (DDAs, UCYN-A), or in the case of heterotrophic diazotrophs, are involved in the breakdown of biomass and CO_2 release. There are ecologically very important differences among different diazotrophs due to the fate of the fixed N (and C). For example, it is assumed that a large share of N fixed by *Trichodesmium* is released as NH_4^+ or DON, and is thus channeled through the dissolved pool/the microbial loop in the mixed layer (Glibert and Bronk, 1994; Mulholland et al., 2004, 2006). In contrast, DDAs have been typically classified as being fast-sinking and thus important in sequestering N and C in the deep ocean (Scharek et al., 1999a, b; Subramaniam et al., 2008; Karl et al., 2012). The unicellular diazotrophs (UCYN-A, *Crocosphaera*) have usually been assumed to mainly channel through the microbial loop, since they are small and thus subject to grazing (Mulholland, 2007). However, with the discovery that UCYN-A is a symbiont of a larger, possibly calcifying eukaryotic alga, the situation appears more complex and the fate of N from organisms such as UCYN-A is unknown. Similarly, *Crocosphaera* strains are different, some of which produce large amounts of exopolysaccharides (Bench et al., 2011; Sohm et al., 2011), which should promote the aggregation and sinking from the surface layer.

Apart from the scarce knowledge about the relative contributions of different species types to N_2 fixation, and in addition to the already-mentioned difficulties in quantifying oceanic N_2 fixation on different temporal and spatial scales, there is very little knowledge about how these diazotroph inputs are coupled with C cycling. Among the photosynthetic diazotrophs, *Trichodesmium* has been studied the most (Mulholland et al., 2006; Mulholland, 2007), and direct CO_2 fixation measurements vary dramatically, with no consistent stoichiometric relationship to the amount of fixed N; this makes it difficult to infer, for example, C fluxes from N_2 fixation measurements, or vice versa, using Redfield stoichiometry (Mulholland, 2007). There is no straightforward way to resolve this except to study the physiologies of each individual diazotroph species and relate this knowledge to biogeochemical forcing. In order to study the elemental stoichiometry in novel UCYN-A symbiotic associations, additional studies are required to understand the ecology of the host species and the physiological regulation of N_2 fixation between the symbiotic partners under different settings. The factors regulating N_2 fixation in free-living and symbiotic diazotrophs, trophic interactions, and the community ecology, in general, will all influence the fate of fixed N and C as well, including the fractions of total diazotroph N inputs that directly fuel either autotrophic or heterotrophic growth.

84.7.2 Coupling of N_2 Fixation to Other Oceanic N Cycle Processes

The complexity of the oceanic N cycle is exemplified by how much we still do not know about the ecology of N_2-fixing microorganisms, despite the immense progress seen in recent years. Consequently, even further away is a good understanding of how different processes in the N cycle are connected and how different nutrient cycles (P, Fe) are interlinked, both at the microscale and at the

scale of ocean basins. One topic of great interest has been to better constrain the main input and loss terms, that is, the interplay between oceanic N_2 fixation and the return flux of N_2 to the atmosphere via denitrification and anammox. Biogeochemical model studies suggested that N_2 fixation and denitrification/anammox should be spatially coupled in the ocean, with active N_2 fixation occurring in relatively cold, partially denitrified, phosphate-rich waters overlying oxygen minimum zones (OMZs), including the Eastern Tropical South Pacific (ETSP) (Deutsch et al., 2007). However, various field campaigns in the ETSP have found very low rates of N_2 fixation, presumably carried out mainly by heterotroph diazotrophs typically occurring at very low abundances (Moutin et al., 2008; Fernandez et al., 2011; Halm et al., 2012). These findings are not in accordance with what the model studies have suggested, and it remains unknown whether heterotroph diazotrophs, or cyanobacteria, episodically occur with higher abundances and fix more N_2, and what energy source heterotrophs use.

84.7.3 Heterotrophic N_2-Fixing Bacteria

Apart from the difficulties in studying heterotroph diazotrophs *in situ* (low abundances, hard to distinguish from contaminants), it is known that they are present in the ocean, and that they express *nifH* (Bostrom et al., 2007; Farnelid et al., 2013). Heterotroph diazotrophs have also been previously cultivated from oceanic surface waters (Maruyama et al., 1970; Werner et al., 1974; Guerinot and Colwell, 1985). However, little is known about the physiology and *in situ* N_2 fixation rates of any specific heterotrophic bacteria. It is difficult to determine the role of heterotrophic bacteria partially because they have usually been studied in the same habitats where cyanobacteria occur (euphotic zone, oxygenated surface waters); environments which appear rather unfavorable for a heterotroph N_2 fixer that presumably needs low O_2 conditions and an adequate supply of carbon for energy. In the upper water column of the oligotrophic oceans, such habitats exist within suboxic microzones of particles, or within zooplankton guts (Braun et al., 1999) or associated with other plankton (Farnelid et al., 2010), but whenever single *nifH* phylotypes are quantified they usually are found in very low abundances relative to cyanobacterial diazotrophs, with few exceptions (Church et al., 2005a). It could be possible that individual phylotypes are very rare but that the sum of many single phylotypes is a quantitatively significant community. The possibility that heterotroph diazotrophs could be symbionts has also been suggested in a number of cases, based on microscopic observations (Farnelid et al., 2010) or on detection of the organism or their *nifH* genes in larger size fractions (Guerinot and Colwell, 1985; Bombar et al., 2013), but a conclusive identification of a symbiosis is lacking. A symbiotic lifestyle would be a possibility for heterotroph diazotrophs to obtain a carbon source for energy from a photosynthetic host, but would also present an O_2 problem.

There are a number of recent reports of heterotroph diazotrophs in OMZs, for example, off Peru, in the Arabian Sea, in the Southern California Bight (Fernandez et al., 2011; Hamersley et al., 2011; Jayakumar et al., 2012), and in the aphotic, anoxic chemocline of the Baltic Sea, where presumably anaerobic heterotroph diazotrophs can reach quite high abundances (up to 10^7 *nifH* gene copies per liter (Farnelid et al., 2013)). It remains to be shown whether these organisms play a significant role in N_2 fixation in these environments, which obviously have more favorable conditions for such diazotrophs and do not contain cyanobacteria. So far, rates measured are relatively low, such as in the Baltic Sea deep waters (Farnelid et al., 2013). Cultivated isolates have been obtained from the Baltic Sea as well (Bostrom et al., 2007; Farnelid et al., 2014), and thus in the near future, genome analyses and physiological experiments might help to better determine their ecological niche.

84.8 CONCLUDING REMARKS

Studies of well-known diazotrophs such as *Trichodesmium*, the recent discoveries of new diazotroph groups, improvements in mathematical modeling and sampling techniques, and increased sampling coverage of the ocean have all improved our understanding of the global significance of oceanic N_2 fixation. While oceanic N_2 fixation undoubtedly plays a key role in global N cycling, challenges remain in answering even basic questions about the organisms and their distributions, the factors that control their abundances and activity, community interactions, and the fate of different diazotrophs in the ocean food webs. The answers to these questions are particularly challenging, since long-term global environmental change, such as ocean acidification, may have many unknown effects on oceanic microbial communities. Comprehensive studies are necessary that combine genomic information, novel sampling techniques, and biogeochemical rate measurements to address questions regarding not only nutrient acquisition but also symbiont–host interactions, grazing, and other ecosystem properties. The continued development and application of new tools and approaches facilitates testing of established paradigms, such as the dominance of oceanic N_2 fixation by *Trichodesmium*, or regulation by abiotic factors such as temperature, oxygen, fixed inorganic N or Fe availability. Methodologies that allow for high resolution- and autonomous detection of key species might help to tackle some of the greatest challenges in achieving adequate sampling coverage, that is, the vastness of the ecosystem at study and the large spatiotemporal variability of the microorganisms inhabiting it. With these new analytical

and instrumentation capabilities, there are likely to be yet more discoveries and surprises that will result to yet more modifications of the paradigms of oceanic N_2 fixation.

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Chapter 85

Requirement of Cell Wall Remodeling for Cell–Cell Communication and Cell Differentiation in Filamentous Cyanobacteria of the Order *Nostocales*

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85.1 INTRODUCTION

Cyanobacteria of the order *Nostocales* and *Stigonematales* are the prokaryotes with probably the most highly evolved multicellular properties, which include functionally differentiated cells in a multicellular network (Maldener et al., 2014) (see Fig. 85.1). According to the classification by Rippka et al. (1979) they are classified in Section IV and V. Recent phylogenetic studies based on whole genome sequences show that these two orders form in fact one monophyletic branch (Subclade B1) within the tree of cyanobacteria (Shih et al., 2013). The defining property of this subclade is the formation of multicellular filaments comprising nitrogen-fixing heterocysts (Fig. 85.1). The two orders differ in one morphological feature: formation of true branching is a specificity of the *Stigonematales* (section V), whereas the filaments of *Nostocales* are non-branched (section IV). Only species of the *Nostocales* have so far been used for molecular studies of multicellular development due to the availability of genetic tools (Cohen et al., 1994; Thiel and Wolk, 1987). In particular, the strain *Anabaena* (*Nostoc*) sp. PCC 7120, also known as *Nostoc* sp. PCC 7120, has been widely used to study heterocyst development (Kumar et al., 2010). The strain *Nostoc punctiforme* ATCC 29133 (in the following *N. punctiforme*) has attracted attention due to its complex life style, comprising the ability of symbiotic interactions with various plants and complex differentiation properties (Meeks et al., 2002), and genetic tools for this strain have been developed. For these practical reasons, the following studies on the role of cell-wall modification in multicellular development in the cyanobacteria have been mostly performed with the strains *N. punctiforme* and *Anabaena* sp. PCC 7120.

85.2 REQUIREMENT FOR CELL WALL MODIFICATION DURING HETEROCYST DIFFERENTIATION AND DIAZOTROPHIC GROWTH

Nostoc sp. forms filaments of hundreds of metabolically interacting cells, surrounded by a continuous Gram-negative outer membrane (OM) (Flores and Herrero, 2010; Flores et al., 2006; Lehner et al., 2011; Schneider et al., 2007; Wilk et al., 2011). Under nitrogen-limiting conditions, about 5–10 % of the cells differentiate in a semi-regular pattern into heterocysts, which are specialized cells that provide the micro-oxic compartment necessary for nitrogen fixation. A remodeling of the cell wall seems to be a requisite for the formation of the special envelope of heterocysts. This

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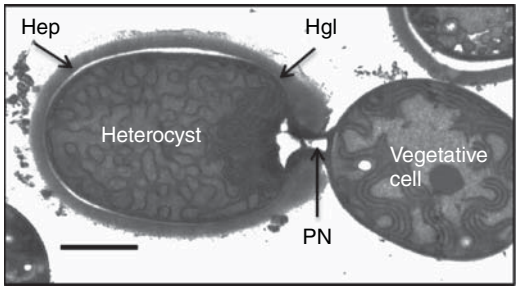

Figure 85.1 Electron micrographs of ultrathin sections of chemically fixed filaments of *Anabaena* sp. PCC 7120 showing the morphological changes in heterocysts: narrow polar neck (PN), envelope of 2 layers: Hgl = glycolipids, Hep = polysacharides. Scale bar = 1 μm.

envelope consists of an outer polysaccharide layer (Hep in Fig. 85.1) and an inner glycolipid layer (Hgl in Fig. 85.1), the barrier for the entrance of oxygen from outside (Wolk et al., 1994). It has been speculated that the transfer of the envelope building blocks from the cytoplasm to the surface requires remodeling of the Gram-negative cell wall, which consists of the peptidoglycan (PG) layer and an outer membrane (Huang et al., 2005; Léganes et al., 2005). Specific transport complexes, such as the DevBCA transporter for the heterocyst glycolipids (Staron et al., 2011), contain periplasmic-spanning channel proteins, which transverse the PG layer and connect to the TolC porine.

Furthermore, the differentiation of heterocysts involves extensive alterations in the shape of the developing cells, in particular in the so-called neck-region connecting to the vegetative cells (PN in Fig. 85.1). Since the shape of the PG sacculus determines the morphology of the cells, it is a necessary consequence that the PG sacculus has to be modified during heterocyst development. It is therefore not surprising that several genes encoding cell-wall processing enzymes are needed for heterocyst development. In *Anabaena* sp. PCC 7120, the following such genes have been identified to be required for heterocyst differentiation: *pbp*2, *pbp*3, *pbp*6, and *hcwA* (Huang et al., 2005; Lázaro et al., 2001; Léganes et al., 2005; Zhu et al., 2001). A mutant in the *conR* gene of *Anabaena* sp. PCC 7120, bearing a LytR-CpsA-Psr domain related to septum formation and cell wall maintenance, cannot grow on N_2 although nitrogenase activity develops. In this mutant the septum between the cells seems to be defective. The protein could be involved in the regulation of septal muramidases (Fan et al., 2005; Mella-Herrera et al., 2011). The functional characterization of other cell wall proteins of *Anabaena* sp. PCC 7120 revealed that they are involved in heterocyst differentiation and function (Nicolaisen et al., 2009). Several PG-binding and cell wall-related proteins were identified, which are highly upregulated under the control of the heterocyst regulator HetR in *Anabaena* sp. PCC 7120 (Mitschke et al., 2011).

Once a functional heterocyst is formed, the products of nitrogen fixation have to be delivered to the neighboring vegetative cells, and conversely, the heterocysts derive carbohydrates from their neighbors (Wolk et al., 1994). The route of the molecules through the individual cell envelopes, which results in highly efficient metabolite and signal exchange between the diverse cells along the filament, has not yet been clarified (Flores and Herrero, 2010; Kumar et al., 2010). In addition to these problems, formation of other types of differentiated cells in *Nostoc* strains, such as the motile hormogonia and the spore-like akinetes, involves structural changes in the cell envelope. A key issue in these processes is the understanding of the role of the PG cell wall. Unfortunately, the structure, biogenesis, and metabolism of PG in cyanobacteria in general, and of the *Nostocales* in particular, have been poorly investigated. For example, until recently it has not been studied how in the multicellular cyanobacteria the cell wall is organized in particular in the region of the septum between neighboring cells. Recently, we set out to address this question and the current review summarizes the essence of these studies.

85.3 THE PG LAYER OF FILAMENTOUS CYANOBACTERIA

The PG of Gram-negative bacteria is a large heteropolymer that consists of linear glycan strands of alternating β-1-4 linked *N*-acetylmuramic acid and *N*-acetylglucosamine, which are connected by peptide bridges of four amino acids. Although the precise architecture of the arrangement of the strands toward each other is still debated (Turner et al., 2013; Vollmer and Seligman, 2010), it is clear that this macromolecule entirely surrounds the cells and determines the cell shape and the stability against turgor pressure of the cells (Margolin, 2009; Vollmer and Bertsche, 2008). In typical Gram-negative bacteria the PG is located between the cytoplasmic membrane (CM) and the OM and has an average thickness of 2,5 nm. Interestingly, cyanobacteria, which belong to the Gram-negative bacteria, have a much thicker murein, ranging from 10 to 700 nm, depending on the strain (Schleifer and Kandler, 1972). Diverse PG synthases and PG hydrolases are involved in PG turnover during cell growth and cell division (Vollmer et al., 2008). At the site of cell division, constriction of the cell goes along with the synthesis of septal PG and the simultaneous formation of two new cytoplasmic membranes.

A first step in cell division in unicellular cyanobacteria as well as in other Gram-negative bacteria is the organization of the divisome machinery at the prospective division site of the cell by the FtsZ ring (Adams and Errington 2009; de Boer, 2010; Mazouni et al., 2004; Miyagishima et al., 2005; Sakr et al., 2006), followed by a constriction of the new septum toward the middle of the cell.

This constriction results in a diminishing annulus in the center of the new septum and finally the CM meeting in the center and separating. Simultaneously, the PG layer is formed by *de novo* synthesis of PG, resulting in one PG layer shared between the originating daughter cells. In *E. coli*, cell division is achieved by splitting of the newly synthesized septal PG from the outside to the center of the septum. Concomitantly with this, newly made OM invaginates into the septum where it is attached to the cleaved PG layers, and finally closes the annulus in the center of the septum (de Boer, 2010). In the process of cleavaging the septal PG, three paralogs of the autolytic enzyme LytC-type N-acetylmuramyl-L-alanine amidase (Pfam: amidase_3) play a prominent role: AmiA, AmiB, and AmiC (Uehara and Bernhardt, 2011; Uehara et al., 2010; Vollmer et al., 2008). Mutants lacking all three amidases fail to split the septal PG and grow in long chains (Heidrich et al., 2002). Interestingly, filamentous cyanobacteria of the order *Nostoc* possess several genes encoding homolog hydrolases, although they apparently do not separate daughter cells following cell division. The function of these amidases is therefore enigmatic, in particular, since it is unknown whether the PG in the septum is split in two layers or remains as a single layer that mechanically connects the neighboring cells. As detailed below, this issue has recently been debated.

The last step of cell division, invagination and fusion of the OM, does not occur during cell division within the filaments of *Nostocales*. Instead, the OM encompasses all cells of the filament without entering the septum, forming a continuous encasement (Flores et al., 2006). Thus, the individual cells possess their own CM, but it is not clear whether cleaving of the PG layer by hydrolytic enzymes and partition between the two cells occurs. In some electron micrographs of chemically fixed filaments, one dark line in the middle of the septum between the CMs of the adjacent cells appears to represent one common PG layer (Flores et al., 2006; Lehner et al., 2013). In contrast, a recent study using the polysaccharide stain ruthenium red revealed two electron dense lines adjacent to the outer face of each CM, and this has been interpreted as representing two separated PG layers (Wilk et al., 2011). To solve the problem, we isolated the pure PG sacculi from filaments of *Anabaena* sp. PCC 7120 and *N. punctiforme* ATCC 29133, respectively, devoid of any proteins and investigated their structure in the light microscope and by TEM. If the septal PG was split, each cell should be surrounded by its own sacculus. Upon sacculus preparation, the filaments should disintegrate into sacculi of individual cells. However, this was not the case. Instead, the sacculi retained the filamentous structure (Fig. 85.2) and the PG was connected at the septum. The filamentous sacculi were not broken at the cross walls but at the thinner lateral sidewalls. A similar result has already been suggested by light micrographs of isolated sacculi of *Anabaena cylindrica* (Dunn and Wolk, 1970).

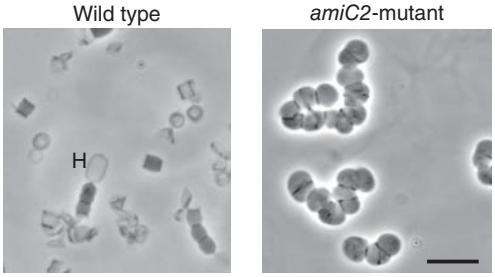

Figure 85.2 Light micrographs of purified sacculi of *N. punctiforme* wild type and the *amiC2* mutant strain: The peptidoglycan represents the shape of the filaments. The peptidoglycan is continuous and not distributed between adjacent cells. Fragmentation of the PG took place during preparation, mostly in the lateral walls. The cell wall of the mutant strain appears more rigid and stable compared to the wild type strain. H = heterocyst. Scale Bar = 10 µm. *Source*: Micrographs by Josef Lehner.

In our TEM analysis, when sacculi were applied in total on coated copper grids, the septa were clearly visible as circular discs defined by a dark ring, which was very stable during sample preparation (see Fig. 85.3). In contrast, the lateral walls were more lightly stained and more labile, resulting again in disruption of the filaments at these sites (Lehner et al., 2013) (Figs. 85.2 and 85.3).

Since "unicellular" sacculi were rarely detected, the PG is not split in the septum as in cell division of unicellular bacteria. In unicellular bacteria, PG splitting is coordinated with OM invagination, suggesting that these processes are interconnected. However, as stated above, the OM does not invade the septum in the *Nostocales*, which is in agreement with the maintenance of a single layered septal PG. Further insights into the cell wall structure of *Nostocales* was obtained by staining the sacculi and the living filaments of *N. punctiforme* with fluorescent vancomycin, which specifically attaches to the non-cross-linked peptides of PG. Using this dye, one common PG layer in the septum could also be visualized. As shown in Figure 85.4, strong staining of the septal PG ring occurred. The non-cross-linked peptide residues that are stained by fluorescent vancomycin are present in those parts of the cell wall where PG synthesis takes place: this is the septal cross wall during cell division. In summary, we propose that the PG sacculus is a macromolecule that surrounds and connects all cells of the filaments with a single layered septal PG that forms a rigid discoidal structure.

85.4 THE NANOPORE ARRAY OF SEPTAL MUREIN

In addition to identifying the overall structure of the PG sacculus of the *Nostocales* filaments, we could identify in those electron micrographs of isolated sacculi an array of

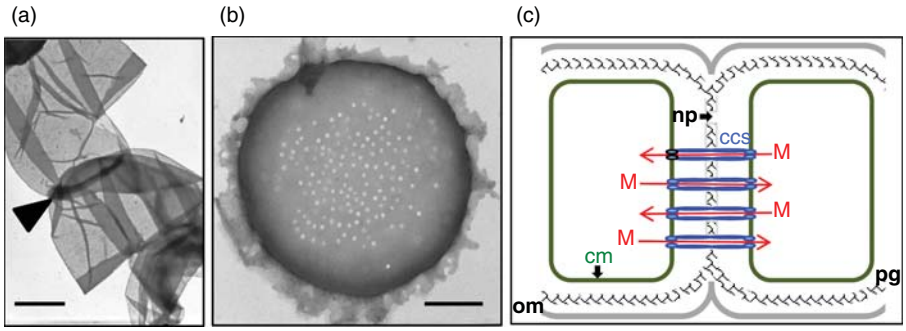

Figure 85.3 The nanopore array of the septal peptidoglycan: electron micrographs of purified sacculi of *N. punctiforme*. A septum is indicated by an arrow head in (a). (b) Isolated septal PG showing the nanopore array. (c) Model of a cross section through the septum between two cells of a filament. np, nano pore; ccs, cell communication structure; M, metabolite; cm, cytoplasmic membrane; pg, peptidoglycan layer; om, outer membrane. Red arrows show transfer of molecules through ccs, anchored in the cm and traversing as protein complexes in the septum through the nanopores with large extracellular domains. Scale bar = 1 µm in (a) and = 300 nm in (b). *Source*: Electron micrographs by Josef Lehner.

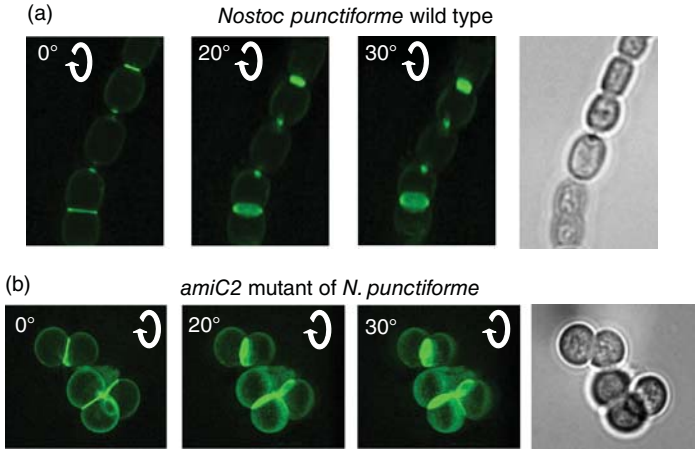

Figure 85.4 *In vivo* fluorescence staining of filaments of *N. punctiforme* wild type strain (a) and the *amiC2* mutant strain (b). Vancomycin, VanFL, strongly labeled the disk-shaped septa in both strains, indicating deposition of new cell wall material and unlinked residues preferentially at the septa. The fluorescence images were created by a deconvolution technique. 0°, 20° and 30° show rotation angles of the image. Bar = 10 µm. *Source*: Micrographs by Josef Lehner.

nanopores in the center of the septal disc (see Fig. 85.3). In addition, in the lateral walls small pores were visible; however, in contrast to the septal array, they were much smaller and irregularly spaced. Similar pores are present in the PG of other bacteria (Turner et al., 2013). The nanopore array, instead, is comprised of roughly 140 nanopores, each 20 nm wide. In some of the pores, dark material was visible, which could be the remnants of non-hydrolyzed PG or proteins, which had escaped chymotrypsin digestion during sacculi preparation (Lehner et al., 2013).

The physiological significance of this nanopore array was revealed by the analysis of mutants in cell-wall hydrolases. We had created mutants of *Anabaena* sp. PCC 7120 and *N. punctiforme* by site-directed mutagenesis of open reading frames *alr0092* (*amiC*1_{Ana}) and NpF1846 (*amiC2*), respectively (Berendt et al., 2012; Lehner et al., 2011). Because of their sequence homology to the cell division protein *N*-acetylmuramyl-L-alanine amidase of *E. coli* (Pfam: amidase_3), these genes were named AmiC. The *Nostoc* AmiC2 mutant strain showed a severe morphological phenotype already under standard growth conditions: Cells were irregularly shaped and the plane of cell division was displaced. Instead of linear filaments, this mutant grows in clumps with clover-leaf-like cell clusters (Fig. 85.4). Furthermore, this mutant is unable to differentiate special cell types (heterocysts, akinetes, and hormogonia) (Lehner et al., 2011). The *Anabaena* AmiC1 mutant strain grew normally as long filaments on a source of combined nitrogen, but was also unable to differentiate heterocysts and hence to grow on N_2 as the sole nitrogen source (Berendt et al., 2012). Since several amidase genes are present in the genomes of these strains, we cannot exclude redundancy with respect to filament morphology in the case of the *Anabaena* mutant. Interestingly, both mutant strains were not able to transport the fluorescent dye calcein in FRAP (fluorescence recovery after photobleaching) studies, indicating lack of cell–cell communication (Mullineaux et al., 2008). Sacculi were prepared from these mutants and analyzed similarly to those of the wild type (see above). Importantly, no nanopores were visible in the septal discs of both mutants (Lehner

et al., 2012, 2013). This indicates that cell wall amidases $AmiC1_{Ana}$ and AmiC2 are involved in the hydrolysis of the PG at specific sites where the nanopores are formed. In other words, they drill holes in the septum, resulting in functional filaments capable of exchanging molecules between cells.

The pores themselves cannot be sufficient to mediate cellular communication, since the CM is closed on both sides of the septum and does not penetrate the septum, as shown clearly in electron micrographs. Instead, the nanopore array in the septal PG could be the framework for cell–cell joining or communication structures, which were suggested in previous studies. Previous ultrastructural analysis of filamentous cyanobacteria revealed connecting structures, which were termed "microplasmodesmata" (Giddings and Staehelin, 1978, 1981; Lang and Fay, 1971; Wildon and Mercer, 1963). The numbers and average diameter of the pores/microplasmodesmata in the studies by Giddings and Staehelin were in the range of our estimations with *N. punctiforme* and *Anabaena* sp. PCC 7120. The nanopore array, as visualized now by the sacculus preparation (Fig. 85.3), provides the first complete view on the arrangement of the connecting structures on the entire septum. Concerning the nomenclature of these structures, we prefer the more general term cell–cell joining structures, since the molecular architecture and composition is not known yet. The term "microplasmodesmata" would imply that the joining structures are direct plasma bridges, delimited by CM, which is, however, not the case (see above).

Candidate channel proteins that are embedded in the nanopores and could form a cell-joining complex are the Fra proteins, which were characterized in *Anabaena* sp. PCC 7120. FraG (also known as SepJ), FraC, and FraD have been localized to the septum of *Anabaena* sp. PCC 7120; mutants of their genes could not grow diazotrophically and their filaments fragmented (Nayar et al., 2007; Flores et al., 2007; Merino-Puerto et al., 2010, 2011; Mariscal et al., 2011). Importantly, these mutants were impaired in molecule exchange between the cells, implying that all three proteins together or separately could form a cell–cell joining complex.

In conclusion, it appears that the cell-wall amidases play a key role in the evolution of multicellular cyanobacteria. These enzymes, which normally are responsible for daughter cell separation, have altered their function: instead of splitting the daughter cells, they perform a fine-tuned remodeling of the septal PG by drilling holes the for insertion of a cell-joining machinery (see model in Fig. 85.3). This structure can be regarded as a prokaryotic organelle for direct cell–cell communication and metabolite exchange. Future studies will focus on the analysis of the septal PG and the mode of function of the amidases in the formation of the nanopore array in *Nostocales*, in context with the Fra-proteins and other factors that conduct the precise and located amidase activity.

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Chapter 86

Nitrogen Fixation in the Oxygenic Phototrophic Prokaryotes (Cyanobacteria): The Fight Against Oxygen

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86.1 CYANOBACTERIA AND NITROGEN FIXATION

Cyanobacteria represent a phylogenetically coherent group of organisms that are characterized by their ability to perform oxygenic photosynthesis (Giovannoni et al., 1988). Predecessors of extant cyanobacteria evolved oxygenic photosynthesis more than 2.4 billion years ago (Knoll, 2008), and therefore the history of cyanobacteria parallels in a substantial part the history of life on Earth. In oxygenic photosynthesis, two photosystems, PSI and PSII, work in series to transfer electrons from water to the universal metabolic electron donors, ferredoxin and NADPH (DeRuyter and Fromme, 2008). Splitting of water in a specialized complex (the Oxygen Evolving Complex) that is adjacent to PSII releases oxygen as a byproduct. In all cyanobacteria studied to date but the special *Gloeobacter violaceus*, the photosynthetic reactions occur in intracellular membranes termed thylakoids. This implies that in an oxic environment, the cytoplasm of cyanobacteria not only contains oxygen that may diffuse from the atmosphere into the cell but additionally contains oxygen produced inside the cell. This poses a tremendous burden to those cyanobacteria that perform nitrogen fixation, which is carried out by the extremely oxygen-sensitive enzyme nitrogenase (Gallon, 1981; Fay, 1992; see Chapter 1).

In spite of the unifying character of oxygenic photosynthesis, disparate morphologies are displayed among cyanobacteria, which can be found in an organization ranging from single cells to complex filaments made of long chains of interconnected cells. In a classic taxonomy of cyanobacteria based on the study of pure microbial cultures, Rippka et al. (1979) classified these organisms into five groups termed Sections. Sections I and II comprise unicellular cyanobacteria, in which those belonging to Section I reproduce by binary fission or by budding and those of Section II by multiple fission or by both multiple and binary fission. Sections III, IV, and V include filamentous cyanobacteria, that is, cyanobacteria that make trichomes (chains of cells) that grow by intercalary cell division and reproduce by random trichome breakage or, in some cases, by formation of hormogonia or germination of akinetes. While the filaments of Section III cyanobacteria are composed only of vegetative cells, the trichomes of Section IV and V cyanobacteria contain heterocysts specifically when the organisms grow lacking any source of combined nitrogen, and some strains also form hormogonia or/and produce akinetes (Rippka et al., 1979). While in Sections III and IV cell division occurs only in one plane (perpendicular to the filament axis), in Section V cyanobacteria cell division can take place in more than one plane producing truly branched filaments. Nonetheless, Sections IV and V together comprise a monophyletic group

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of organisms implying that heterocysts, whose presence is a defining taxonomic character, arose only once in evolution (Giovannoni et al., 1988). It is now well established that heterocysts are sites of nitrogen fixation (Herrero et al., 2013). The capability to fix atmospheric nitrogen is however widespread among cyanobacteria, being exhibited by organisms in the five taxonomic Sections (Rippka et al., 1979). Thus, non-heterocyst-forming as well as heterocyst-forming cyanobacteria, and among the former, unicellular as well as filamentous strains, express nitrogenase activity (Rippka and Waterbury, 1977). Although cyanobacteria diverged relatively early in evolution (Schirrmeister et al., 2011), the widespread presence of nitrogenase-encoding genes in cyanobacteria comes as no surprise given the very early origin of this enzyme in evolution (Fani et al., 2000).

86.2 PHYLOGENETIC ANALYSIS

Our knowledge of cyanobacteria as a group has significantly improved after the sequencing of numerous cyanobacterial genomes, as is the case for living beings in general. As of this writing, about 150 cyanobacterial genome sequences have been completed (see Dagan et al., 2013; Shih et al., 2013). A phylogenetic tree based on 31 concatenated proteins common to all analyzed cyanobacteria unravels seven major clades (clades A to G) in the phylogeny (Shih et al., 2013; see schematic in Fig. 86.1). Of note is the confirmation that heterocyst formers constitute a monophyletic group (clade B1), and that this group is phylogenetically related to groups (B2, B3, A) in which filamentous cyanobacteria are present, consistent with the notion (obvious in any case) that heterocyst formers evolved from a filamentous cyanobacterium (Swingley et al., 2008). In contrast, Sections I and III are widely polyphyletic, and their characteristic genera, such as Section I *Synechococcus* (defined on morphological grounds), are found scattered in the tree. Indeed, filamentous cyanobacteria are found in most clades (in all but clades E and G), consistent with the idea that the ability to form filaments evolved early in cyanobacterial history, although it was lost and regained several times (Schirrmeister et al., 2011). Also of note is that within clade C, no filamentous cyanobacterium is found in subclade C1, which is constituted by the abundant marine unicellular cyanobacteria of the genera *Synechococcus* and *Prochlorococcus*. Finally, most Section II cyanobacteria cluster together in subclade B2, but one sequenced strain is found in subclade B1.

The distribution of the genes encoding nitrogenase and accessory proteins can be traced in the sequenced genomes (Dagan et al., 2013; Shih et al., 2013). Figure 86.1 shows that cyanobacteria bearing the nitrogenase genes are scattered throughout the phylogenetic tree, although they are not present in clades E and F or in subclades C1 and C2. Very frequent occurrence of nitrogenase genes can be

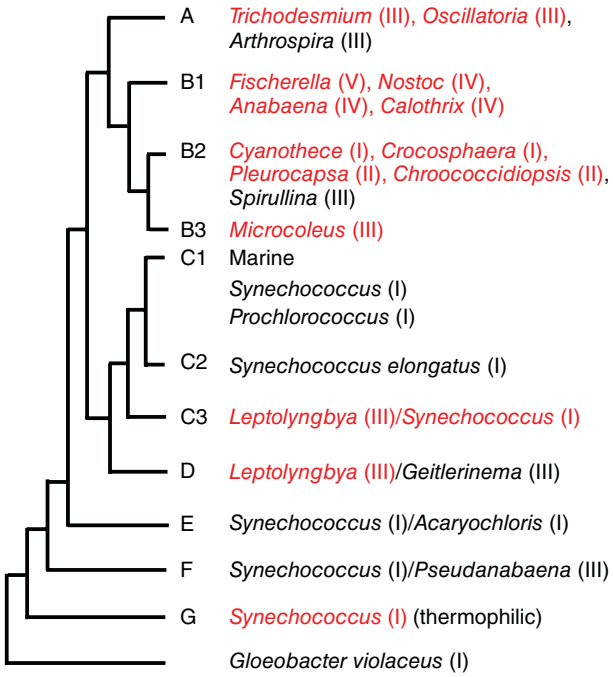

Figure 86.1 Schematic of the phylogenetic tree of cyanobacteria (based on the phylogenetic analysis published by Shih et al., 2013). The tree recognizes seven major clades, with clades B and C further divided in subclades. *Gloeobacter violaceus*, which lacks intracellular thylakoids, is apart from all other known cyanobacteria. Some characteristic genera are indicated for each clade or subclade, and the taxonomic Section of Rippka et al. (1979) to which they belong is indicated in roman numerals (I to V). Nitrogen fixing organisms (demonstrated or predicted from the presence of the *nif* genes in their genomes) are indicated in red. Phylogenetic distances are not depicted.

noticed in the upper part of the tree, which includes clades A and B that contain strains belonging to the five taxonomic Sections. The phylogeny of nitrogenase does not match, however, that of the organisms, likely reflecting that events of horizontal gene transfer have been relatively common in the evolution of cyanobacteria contributing to the current distribution of the capability to fix nitrogen among these organisms (Swingley et al., 2008). The *nif* genes encoding nitrogenase and genes encoding some nitrogen fixation auxiliary proteins are generally found clustered together in the genomes of nitrogen-fixing cyanobacteria (see examples in Fig. 86.2a, b). In some heterocyst-forming cyanobacteria, *nifD* and *fdxN* are interrupted by DNA elements that are removed during heterocyst differentiation (Golden et al., 1991; Carrasco et al., 1994) resulting in *nif* gene clusters that are similar to those of other cyanobacteria (Fig. 86.2c). In these clusters, the presence of the nitrogenase structural genes *nifHDK*, the nitrogenase Fe-Mo cofactor biosynthesis genes *nifEN*, *nifX*, and *nifBSU* (Rubio and Ludden, 2008; see Chapter 7), as well as ferredoxin-encoding genes, is

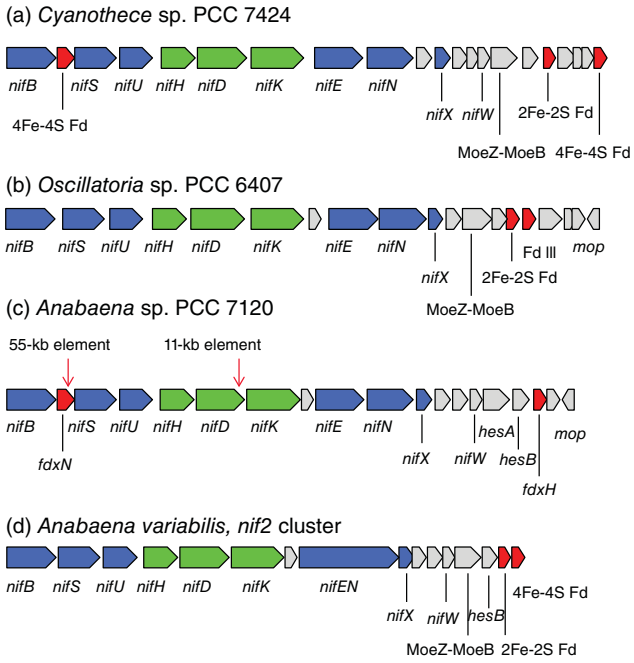

Figure 86.2 Nitrogen fixation gene clusters of cyanobacteria. (a) *nif* gene cluster from *Cyanothece* sp. strain PCC 7424 (redrawn from the genomic sequence; http://img.jgi.doe.gov/). In addition to some identified *nif* genes, there are genes encoding ferredoxins (Fd), a molybdenum cofactor-related protein of the MoeZ-MoeB type and hypothetical proteins. (b) *nif* gene cluster from *Oscillatoria* sp. strain PCC 6407 (redrawn from the genomic sequence; http://img.jgi.doe.gov/). *mop* encodes a molybdopterin-binding protein. (c) *nif* gene cluster from *Anabaena* sp. strain PCC 7120 (drawn from information in the literature; see Böhme, 1998). *hesA* encodes a molybdopterin biosynthesis protein (similar to MoeZ-MoeB proteins) and *hesB* encodes a protein related to iron–sulfur cluster biogenesis. The *nifVZT* genes are in a different chromosome location (see Böhme, 1998). (d) *nif2* gene cluster from *Anabaena variabilis* ATCC 29143 (redrawn from the genomic sequence; http://img.jgi.doe.gov/). This organism also contains a gene cluster (*nif1*) similar to that of *Anabaena* PCC 7120, but lacking the *fdxN* 55-kb intervening element, and *vnf* genes (see the text). Color code: green, nitrogenase structural genes; blue, biosynthesis of the Fe–Mo cofactor genes; red, ferredoxin genes.

evident (Fig. 86.2). Clustering of related genes can facilitate coordinated regulation of gene expression, but it could also reflect that those genes were jointly received as a package (or gene-cassette) from a heterologous biological source.

86.3 STRATEGIES OF NITROGEN FIXATION

In the initial characterization of cyanobacteria, the heterocyst-forming strains proved to be aerobic nitrogen fixers, since they were able to grow readily in the absence of combined nitrogen (Rippka et al., 1979). A few unicellular strains of *Gloeocapsa* (now known as *Gloeothece* sp.) were also observed to grow fixing N_2 aerobically (Rippka et al., 1979). Nonetheless, the question arose whether, as is the case for many free-living nitrogen-fixing bacteria, other strains could fix nitrogen under anoxic conditions. *Plectonema boryanum*, a Section III cyanobacterium, was found to fix nitrogen and grow using N_2 as the nitrogen source when incubated in a gas phase without added oxygen, indicating that there are anaerobic fixers, or perhaps organisms capable of nitrogen fixation under micro-oxic conditions, among cyanobacteria (Stewart and Lex, 1970). Rippka and Waterbury (1977) developed a test in which the cells, after being forced to accumulate glycogen, were subjected to strict anaerobic conditions (including inhibition of PSII by DCMU) and checked for the ability to reduce acetylene to ethylene as an indication of nitrogenase activity. The expression of nitrogenase under such anaerobic conditions was found widespread among the cyanobacteria (Rippka and Waterbury, 1977), consistent with the presence of nitrogen fixation genes widespread in the phylogenetic tree of cyanobacteria as discussed above. However, an important question is whether these cyanobacteria that can fix N_2 under artificial anoxic conditions do so in natural habitats, such as anoxic sites that may exist within some microbial mats, or are somehow able to fix N_2 aerobically. Some cyanobacteria that may fix N_2 anaerobically are those of the genus *Oscillatoria* that bear nitrogen fixation genes and can perform anoxygenic photosynthesis, which is based on inhibition of PSII by H_2S, which provides electrons to PSI (Padan, 1979).

86.3.1 Temporal Separation

Determination of nitrogenase activity (acetylene reduction) and photosynthetic oxygen evolution in batch cultures of *Gloeothece* sp. or *P. boryanum* (under micro-oxic conditions for the latter) showed that nitrogenase and photosynthesis were highest at different times in the cultures (Gallon et al., 1974; Misra and Tuli, 2000), indicating that a temporal separation of both processes could be a mechanism to make them compatible. Introduction of diel light–dark periods in these studies further clarified this mechanism, since nitrogenase activity was found mainly in darkness whereas photosynthesis was, of course, restricted to the light periods (Gallon, 1981). This temporal separation of nitrogenase activity and oxygenic photosynthesis in non-heterocyst-forming cyanobacteria has been amply documented thereafter in both unicellular and filamentous cyanobacteria (see, e.g., Stal and Krumbein, 1985; Mitsui et al., 1986; Colón-López et al., 1997; Compaoré and Stal, 2010; Mohr et al., 2013).

Because nitrogen fixation requires a reductant and energy in the form of ATP, the question arises of how nitrogenase is metabolically supported. In *Cyanothece*, nitrogenase activity requires respiration rather than photosynthesis, which is manifest in a strict dependence on O_2

levels that have to be high enough to support respiration but not as high as to inhibit nitrogenase (Maryan et al., 1986). Indeed, nitrogenase activity and respiration coincide in the dark period of light–dark cycles (Colón-López et al., 1997). Respiration has the additional role of decreasing the intracellular concentration of O_2, which is reduced by terminal respiratory oxidases including cytochrome *c* oxidases, in order to diminish damage to nitrogenase (Toepel et al., 2008). Respiration in turn depends on carbohydrate accumulated in the form of glycogen during the period of photosynthetic activity (Mitsui et al., 1986; Schneegurt et al., 1994). *Cyanothece* sp. strain ATCC 51142 is an aerobic N_2-fixing unicellular cyanobacterium in which detailed gene expression, transcriptomic and proteomic studies have been performed (Colón-López et al., 1997; Toepel et al., 2008; Aryal et al., 2011). It is of interest for our discussion that these studies have shown that photosynthesis, ATP synthase, CO_2 fixation, and glycogen biosynthesis genes are expressed in the light period of light–dark cycles. In contrast, glycogen degradation, respiration, and *nif* genes are expressed entering the dark period (in the case of the respiration genes) or within the dark period. Soon after nitrogenase peaks in this period, a nitrogen reserve material, cyanophycin (multi-L-arginyl-poly-L-aspartic acid), accumulates, which is subsequently utilized during the light period (Li et al., 2001).

Studies on thermophilic, aerobic N_2-fixing unicellular *Synechococcus* spp. in a natural site (a microbial mat) have shown a rise in *nif* transcripts in the evening, with a subsequent decline during the night (Steunou et al., 2008). Interestingly, however, the level of the NifH polypeptide remained stable during the night and only declined when the mat became oxic in the morning. Thus, it is well established that under natural diel cycles, many nitrogen-fixing, non-heterocyst-forming cyanobacteria separate nitrogen fixation and photosynthesis in time. Conditions can be established, nonetheless, for example by manipulating oxygen levels, under which some of these cyanobacteria can fix nitrogen under illumination (see, e.g., Ortega-Calvo and Stal, 1991; Compaoré and Stal, 2010), and variations in the rhythms of respiration and nitrogen fixation have been noted between related cyanobacterial strains (Bandyopadhyay et al., 2013).

In diel light–dark cycle studies with aerobic N_2-fixing unicellular cyanobacteria such as *Cyanothece* sp. strains PCC 8801 (formerly known as *Synechococcus* sp. strain RF-1) and ATCC 51142, it became clear that when the incubation is changed to continuous light, rhythmicity in the expression of *nif* genes and some other genes is maintained to some extent (see, e.g., Mitsui et al., 1986; Colón-López et al., 1997; Huang et al., 1999). These observations imply a circadian regulation of the expression of genes relevant for diazotrophy in these aerobic N_2-fixing cyanobacteria, and as many as 10% of the genes in the genome of a cyanobacterium such as *Cyanothece* sp. strain ATCC 51142 exhibit a circadian behavior (Toepel et al., 2008). Thus, the oscillation required to keep nitrogenase activity restricted to periods in which photosynthesis is not operative appears to be connected to the circadian clock. It is of note that circadian control has been investigated in depth in a unicellular cyanobacterium, *Synechococcus elongatus* (Dong et al., 2010).

86.3.2 Spatial Separation

Different physiological behaviors are found concerning the expression of nitrogenase activity among non-heterocyst-forming filamentous (Section III) cyanobacteria (Fay, 1992; Bergman et al., 1997). Nonetheless, some strains belonging to this group show the diel variation described above for unicellular aerobic N_2 fixers confining nitrogenase activity to the dark period (see, e.g., Stal and Krumbein, 1985). A strikingly different organism is, however, *Trichodesmium* sp., a globally important oceanic N_2 fixer (Bergman et al., 2013; see Chapter 84). Direct comparison shows that, in contrast to *Cyanothece* nitrogenase activity that peaks in the dark, *Trichodesmium* nitrogenase activity peaks during the light period of a light–dark cycle (see Fig. 2 in Bergman et al., 1997). *Trichodesmium* does not produce heterocysts, making its capability to fix N_2 in the light puzzling. Confining nitrogenase expression to some central zones in the filament, in which photosynthesis and CO_2 fixation appear to be downregulated, seems to be the solution (for a recent review, see Bergman et al., 2013). These zones consist of rows of 10 to 20 cells, termed "diazocytes," which have a lighter pigmentation than other cells in the filament and in which a nitrogenase protein such as NifH and a respiratory protein such as cytochrome *c* oxidase can be immunolocalized at increased levels (Bergman et al., 1993). The frequency of diazocytes changes in diel cycles (Sandh et al., 2009). Whether diazocytes are genuine differentiated cells or are cells transitionally devoted to nitrogen fixation is under debate (Bergman et al., 2013; Stal and Zehr, 2008). In addition to this spatial separation, *Trichodesmium* also appears to segregate CO_2 fixation and N_2 fixation during the day, with transient accumulation of fixed nitrogen in cyanophycin (Finzi-Hart et al., 2009).

As is the case with *Trichodesmium*, in studies involving diel light–dark cycles heterocyst-forming cyanobacteria normally fix N_2 in the light (Gallon, 1981). Heterocyst formers represent the paradigm of spatial separation of photosynthesis and N_2 fixation (Flores and Herrero, 2010), which is discussed below. Heterocyst differentiation and the capability to fix N_2 in any type of non-heterocyst-forming cyanobacterium develop only when the organism faces conditions of nitrogen deficiency, lacking any source of combined nitrogen. Thus, two main mechanisms appear to regulate nitrogenase gene expression in cyanobacteria: the circadian control referred

to earlier and nitrogen control. The latter has been mainly investigated in relation to heterocyst differentiation. On the other hand, the detailed studies on control of *nif* gene expression by oxygen that have been done in other nitrogen-fixing organisms have not been done in cyanobacteria.

86.4 NITROGEN FIXATION IN HETEROCYST-FORMING CYANOBACTERIA

A remarkable solution to carrying out the incompatible processes of oxygenic photosynthesis and nitrogen fixation at the same time is the confinement of each activity in a different cell type, as found in the filamentous, heterocyst-forming cyanobacteria. The heterocysts have been shown to contain most, if not all, of the nitrogenase enzyme of a diazotrophic filament and to be the sites of expression of *nifHDK* (reviewed in Herrero et al., 2013; see Fig. 86.3). Under conditions of combined nitrogen scarcity, some cells located at defined positions in the filament differentiate into heterocysts through a complex process that results in cessation of photosynthetic water splitting and expression of the machinery for N_2 fixation. In contrast, in the diazotrophic filament, the vegetative cells keep the capability to perform oxygenic photosynthesis. Therefore, in these cyanobacteria diazotrophic growth depends on the activity of two interdependent cell types: the vegetative cells that perform oxygenic photosynthesis and fix the bulk of CO_2 and the heterocysts that fix N_2. Thus, heterocyst-forming cyanobacteria represent a case of truly pluricellular bacteria with different cell types that are specialized in specific nutritional tasks and contribute to the performance of the filament as the organismic unit (Flores and Herrero, 2010).

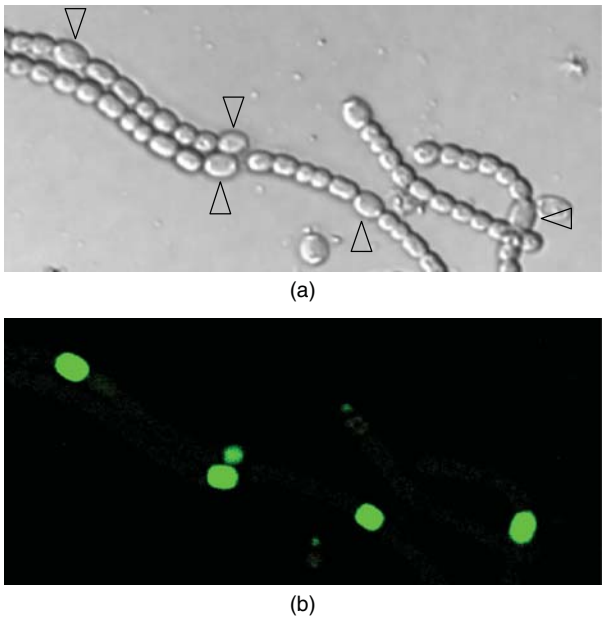

Figure 86.3 Localized expression of a *nif* structural gene in the heterocysts. Bright field (a) and GFP fluorescence (b) micrographs of a filament suspension of a derivative of *Anabaena* sp. strain PCC 7120 bearing in the chromosome the *gfp-mut2* gene fused to the 5′ region of *nifH* (strain CSAM138). The suspension was incubated in $BG11_0$ medium (without combined nitrogen) for 13 h and the GFP fluorescence was visualized by standard methods (see, e.g., Olmedo-Verd et al., 2006). Note that GFP fluorescence originates from heterocysts, which are marked by arrowheads in the upper panel. Strain CSAM138 constructed and micrographs taken by Alicia M. Muro-Pastor in the authors' laboratory.

86.4.1 Intercellular Communication in the Diazotrophic Filament

In relation to the cell specialization established in the diazotrophic filament, growth depends on molecular exchanges between the different cell types. The vegetative cells transfer to the heterocysts fixed carbon in the form of some metabolites, of which sucrose (López-Igual et al., 2010; Vargas et al., 2011) and alanine (Pernil et al., 2010) are likely vehicles that serve as reductant and energy sources, whereas glutamate serves as a skeleton for the incorporation of the ammonium resulting from N_2 fixation (Thomas et al., 1977). In turn, the heterocysts donate to the vegetative cells fixed nitrogen, with some amino acids such as glutamine and cyanophycin-related metabolites (aspartate, arginine, and the aspartyl-arginine dipeptide) being likely nitrogen vehicles (Herrero et al., 2013; Burnat et al., 2014). In addition, signaling molecules, such as a PatS morphogen that is involved in determining the pattern of heterocyst distribution along the filament, are also intercellularly transferred (Yoon and Golden, 1998; Corrales-Guerrero et al., 2013).

The precise mechanism(s) of intercellular molecular exchange are unknown, but recent work has identified possible paths of transfer. The cyanobacteria are diderm bacteria bearing an outer membrane outside of the cytoplasmic membrane and the peptidoglycan layer (Flores and Herrero, 2010). In the heterocyst formers, the outer membrane is continuous along the filament defining a continuous periplasmic space that might represent a communication conduit between the cells in the filament (Mariscal et al., 2007). On the other hand, some channels permitting direct communication between cells that can be probed with fluorescent tracers appear to exist (Mullineaux et al., 2008). In the model heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 (hereafter *Anabaena* PCC 7120), proteins located at the intercellular septa in the filaments have been identified that appear to be part of those channels. These are SepJ (Mariscal et al., 2011) and FraCD (Merino-Puerto et al., 2011), which would constitute two different types of channels. Which molecules are intercellularly transferred

through the periplasmic space (if any) or through each of these proteinaceous channels remains to be established.

86.4.2 The Heterocyst

The differentiation of the cyanobacterial heterocyst is a complex process producing cells with multiple morphological and biochemical differences compared to the vegetative cells. These differences are aimed at increasing the efficiency of the process of N_2 fixation and its protection against external O_2. The heterocyst bears special layers in the cell envelope that are outside of the outer membrane and represent a barrier for the penetration of gases, notably O_2, into the cytoplasm: a layer of specific glycolipids and, external to it, a layer of specific polysaccharides (Walsby, 2007; Flores and Herrero 2010). Indeed, many mutant strains have been isolated, which, as the result of impairment in the synthesis or deposition of any of these layers, lose the ability to fix N_2 under aerobic conditions, although they can fix under anoxic conditions (Herrero et al., 2013). Heterocysts are connected with the neighboring vegetative cells by narrowed septa exhibiting a differentiated structure that allows intercellular molecular exchanges while keeping a minimum surface of cell-to-cell contact, which would hamper oxygen permeation into the heterocyst (Walsby, 2007).

Regarding metabolism, associated to conspicuous reorganization of the internal thylakoid membranes, the heterocysts lack activity of oxygenic photosynthesis although they exhibit photosynthetic activity based on PSI. They also lack ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and thus photosynthetic CO_2 fixation, which enables directing the available ATP and reductant to the reduction of N_2. On the other hand, heterocysts have increased levels of sugar catabolic enzymes including a specific invertase, InvB (López-Igual et al., 2010; Vargas et al., 2011), enzymes of the oxidative pentose phosphate pathway such as glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, and specific terminal respiratory oxidases such as Cox2 and Cox3 (reviewed in Herrero et al., 2013). The increased respiratory activity of the heterocysts is related to the metabolism of sugars such as sucrose received from the vegetative cells, and serves to scavenge traces of O_2 that could have penetrated into the cells. The heterocysts also have a specific membrane-bound (NiFe)-uptake hydrogenase that recycles H_2 produced in the nitrogenase reaction and contributes, through respiration, to O_2 scavenging (Bothe et al., 2010). Additional mechanisms to protect nitrogenase from oxygen or oxygen-reactive species appear to exist. Flavodiiron proteins induced specifically in the heterocyst have recently been described that may protect N_2-fixing enzymes by reducing molecular oxygen directly to water (Ermakova et al., 2013). A rubrerythrin (RbrA) that participates in the reduction of H_2O_2 with NADPH in a reaction that depends on ferredoxin:$NADP^+$ oxidoreductase is expressed in the heterocysts of *Anabaena* PCC 7120, where it has been suggested to protect nitrogenase (Zhao et al., 2007).

In the heterocysts, a number of pathways contribute to the ATP pool used mainly to drive N_2 fixation and the incorporation of the resulting NH_4^+ into C skeletons. ATP can be produced photosynthetically by cyclic photophosphorylation through PSI or by oxidative phosphorylation using H_2, with the concourse of the uptake hydrogenase, or reduced pyridine nucleotides resulting from carbohydrate catabolism. Indeed, a *Nostoc* sp. ATCC 29133 mutant of the *zwf* gene encoding glucose-6-phosphate dehydrogenase fails to grow diazotrophically, stressing the importance of this route (Summers et al., 1995). Electrons from H_2 and reduced pyridine nucleotides can be also used to reduce, either via PSI or through ferredoxin:$NADP^+$ oxidoreductase, a ferredoxin that donates electrons directly to nitrogenase. The *fdxH* gene encodes a heterocyst-specific [2Fe–2S] ferredoxin, which is a principal electron donor to nitrogenase in these cells (Böhme and Haselkorn, 1988; Masepohl et al., 1997). The ammonium resulting from N_2 reduction is incorporated by glutamine synthetase, of which heterocysts bear high activity, into glutamate received from vegetative cells (reviewed in Herrero et al., 2013). In addition to transferring nitrogen to vegetative cells, heterocysts conspicuously accumulate the nitrogen reservoir cyanophycin, which is visible as granules located at the heterocyst poles where it could serve the role of a nitrogen buffer (Sherman et al., 2000).

86.4.3 Nitrogen Fixation Genes in Heterocyst-Forming Cyanobacteria

All heterocyst-forming cyanobacteria studied to date bear nitrogen fixation gene clusters encoding a Mo-dependent nitrogenase system similar to that of other well-studied diazotrophic bacteria (Fig. 86.2c), which is expressed specifically in the heterocysts (see Böhme, 1998). The genes for this conventional Mo-nitrogenase were first identified by heterologous hybridization (see, e.g., Haselkorn et al., 1983) and later by genome sequencing. Nitrogen fixation has been most extensively studied in two strains, the essentially photoautotroph *Anabaena* PCC 7120 and the facultative heterotroph *Anabaena variabilis* ATCC 29413. In *Anabaena* PCC 7120, a recent transcriptomic study has shown that expression of genes in the nitrogenase gene cluster takes place at high levels after 21 h of incubation of the filaments in the absence of combined nitrogen (Flaherty et al., 2011). In *Anabaena* PCC 7120, it had been described previously that, after excision of the 55-kb DNA element from inside *fdxN*, *nifB*, *fdxN*, *nifS*, and *nifU* could be cotranscribed in the absence of combined nitrogen from a transcription start point (TSP) located 189 nucleotides upstream from the *nifB* start (Mulligan and Haselkorn, 1989). After excision of the

11-kb DNA element from inside *nifD*, the *nifH*, *nifD*, *and nifK* genes were also described to be cotranscribed from a TSP located 128 upstream from *nifH* (Golden et al., 1991). Indeed, transcripts that include the messages of *nifH*, *nifHD*, *and nifHDK* are readily detected (e.g., Valladares et al., 2007). Recently, in studies performed in *A. variabilis* the detected transcripts including the messages of *nifHDK* alone have been described to result from processing of larger transcripts that originate from promoters located inside *nifU* and, principally, upstream of *nifB* (Ungerer et al., 2010; Pratte and Thiel, 2014). These results cast doubt on the actual location of the promoter(s) for *nifHDK* expression also in *Anabaena* PCC 7120.

Besides the heterocyst-specific Mo-nitrogenase that is similar to that of *Anabaena* PCC 7120 (Nif1), *A. variabilis* bears another gene cluster (*nif2*; Fig. 86.2d) encoding a second Mo-enzyme system that is expressed in all cells of the filament but only under anoxic conditions (Schrautemeier et al., 1995; Thiel et al., 1995). The molecular mechanism for the possible oxygen control of the expression of the *nif2* gene cluster is unknown. Expression of Nif2 nitrogenase under anoxic conditions appears not to prevent heterocyst differentiation or expression of the Nif1 system, so that both can be simultaneously expressed under those conditions. The Nif2 nitrogenase system is the product of a gene cluster that has an organization similar to that of the *nif1* cluster (Thiel et al., 1997). In addition, *A. variabilis* bears genes similar, although with some differences in their organization, to the *vnfDGK* genes of *Azotobacter vinelandii* encoding a V-dependent nitrogenase (Vnf) (Kentemich et al., 1988; Thiel, 1993). In *A. variabilis*, the genes *vnfG* and *vnfD* are fused into a single gene designated *vnfDG*, and *vnfH* is located in a different genomic region. The *vnfDGK* genes, on the one hand, and the *vnfEN* genes, on the other hand, are expressed in the absence of ammonium and Mo, with or without vanadium, the former ones producing transcripts that would correspond to *vnfDG* and *vnfDGK* (Thiel, 1993, 1996; Pratte et al., 2013). The *vnfEN* genes of *A. variabilis* show higher similarity to the *vnfDK* genes of the same organism than to the *vnfEN* genes of *A. vinelandii*, thus pointing to an origin of the *Anabaena* genes by genomic duplication rather than by lateral transfer together with *vnfDK* (Thiel, 1996). The *nifB* gene in the *nif1* gene cluster, which is expressed irrespective of the presence of Mo and V in the external medium, is required for the activity not only of the Nif1 enzyme, but also of the Vnf nitrogenase (Lyons and Thiel, 1995).

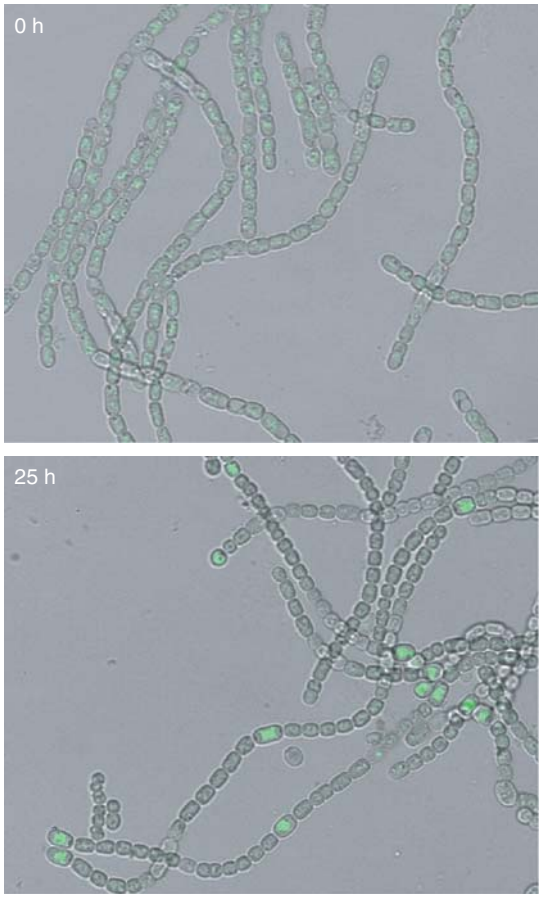

Figure 86.4 Localization of NtcA in filaments of *Anabaena* sp. strain PCC 7120. The *gfp-mut2* gene (stop codon removed) was linked to the 5′ end of *ntcA* (start codon removed) by a sequence encoding a four-Gly linker, producing a gene that encodes a fusion GFP-NtcA protein. *Anabaena* strain CSAL23, in which the *gfp-mut2-ntcA* fusion gene replaces *ntcA*, was grown in $BG11_0$ medium supplemented with ammonium as a nitrogen source (0 h) and subsequently incubated for 25 h in $BG11_0$ medium lacking any source of combined nitrogen. A merge of bright field and GFP fluorescence images is shown. Note that GFP-NtcA can be detected at low levels in all the cells of the ammonium-grown filaments and, upon incubation without combined nitrogen, at increased levels specifically in heterocysts.

86.4.4 Regulation of Gene Expression During Heterocyst Differentiation

The conspicuous structural and metabolic differences between the two cell types of the diazotrophic filament of heterocyst-forming cyanobacteria are the result of a distinct program of gene expression displayed during the developmental process that includes the differentiation of the heterocysts. This program involves the activation or repression of hundreds of genes specifically in the differentiating cells and at specific times throughout the differentiation process (Herrero et al., 2013). The serial gene expression activation is orchestrated by two principal transcriptional regulators, the global regulator NtcA, which is a member of the CRP/FNR family of transcription factors (Herrero et al., 2004; Zhao et al., 2010), and the differentiation-specific factor HetR, which does not exhibit similarity to any other known protein (Buikema and Haselkorn, 1991; Kim et al.,

2011). The differentiation process responds to the external cue of combined nitrogen scarcity and is initiated when NtcA perceives a condition of high carbon-to-nitrogen balance, signaled by high levels of 2-oxoglutarate, which is a positive effector of the regulator (Valladares et al., 2008; Zhao et al., 2010). Mutual activation of the expression of the *hetR* and *ntcA* genes by NtcA and HetR (Muro-Pastor et al., 2002), potentiated by autoregulation (Black et al., 1993; Buikema and Haselkorn, 2001; Olmedo-Verd et al., 2006), leads to increased levels of NtcA and HetR in the differentiating cells. At least the NtcA levels appear to remain high in the mature heterocysts (Fig. 86.4). Accumulation of NtcA and HetR allows for direct induction of some genes during the early and medium stages of differentiation (see, e.g., Camargo et al., 2012; reviewed in Herrero et al., 2013), including genes for other regulators that co-operate with NtcA in the induction of late genes, among them the *nif* genes.

Expression of the genes encoding the N_2 fixation system is integrated in the heterocyst differentiation program, thus responding to the external cue of nitrogen availability. Indeed, *nif* gene expression seems not to be regulated by the intracellular O_2 levels (Elhai and Wolk, 1990), consistent with the fact that *nifHDK* expression is unaltered in mutants of *Anabaena* PCC 7120 lacking one or both of the heterocyst-specific terminal respiratory oxidases, Cox2 and Cox3 (Valladares et al., 2007). Transcription of *nifHDK* requires NtcA (Frías et al., 1994), which can bind *in vitro*, although with low affinity, to sequences in the intergenic region between *nifU* and *nifH* (Valladares et al., 1999). However, if this region were not to include a promoter for *nifHDK* expression, as reported for *A. variabilis* (Ungerer et al., 2010), the role of NtcA binding at this location would be unresolved. The *pipX* gene encodes a small protein only found in cyanobacteria that interacts with NtcA (Llácer et al., 2010). In *Anabaena* PCC 7120 *pipX* is expressed specifically in the cells differentiating into heterocysts at medium-to-late stages of the process, and PipX is required for normal diazotrophic growth (Valladares et al., 2011). In a mutant strain lacking PipX, expression of *nifHDK*, as well as of some other late heterocyst-differentiation genes tested, is severely impaired and retarded with regard to the wild-type strain (Valladares et al., 2011). Thus, PipX is a co-activator of NtcA to promote gene induction during the last steps of differentiation, consistent with a direct positive role of NtcA in *nifHDK* expression. Delayed transcription of *nifHDK* and *fdxH* are also observed in a mutant of the group 2 sigma factor SigE, implicating this sigma factor, which is upregulated late in differentiating heterocysts, in *nifHDK* induction (Mella-Herrera et al., 2011). Unraveling the precise mechanism for activation of the *nif* genes in the heterocyst, including the action of NtcA, PipX, and SigE, will be of much interest in the study of diazotrophy in these unique organisms.

86.5 CONCLUDING REMARKS

The literature summarized in this review indicates that the capability to fix N_2 is widespread in cyanobacteria. Because oxygenic phototrophy is the dominant metabolic mode of these organisms, strategies to make compatible N_2 fixation and oxygenic photosynthesis have been selected in evolution. Temporal separation of the two processes, with an evident circadian regulation that adapts metabolic processes to the appropriate periods of our planet's diel cycle, is widely used. In filamentous cyanobacteria, spatial separation restricting N_2 fixation to some cells in the filament is also found. This strategy permits N_2 fixation when the cyanobacteria are metabolically most active, in the light. In the case of heterocyst-forming cyanobacteria, the strategy of spatial separation has led to the development of one of the simplest multicellular systems known in the living world. Much has been learnt in recent years on these N_2-fixing systems, but some key aspects remain to be explored. These include molecular details of regulation of *nif* gene expression linked to the circadian clock and, in the case of heterocyst formers, to developmental control. In heterocyst formers, structural and functional details of the operation of intercellular molecular exchange in the diazotrophic filament are also major research issues for future research.

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Chapter 87

Underestimation of Marine Dinitrogen Fixation: A Novel Method and Novel Diazotrophic Habitats

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87.1 INTRODUCTION

Biological N_2-fixation is the largest source of fixed-nitrogen input into the ocean and controls marine primary productivity on geological timescales (Falkowski, 1997; Duce et al., 2008). Yet, estimates indicate a greater total oceanic N-loss than N-gain when extrapolated from discrete measurements (Codispoti, 2007; Deutsch et al., 2007). This discrepancy could be ascribed to either an overestimation of N-loss, which even with the most conservative calculations has been considered to be unlikely; or to an underestimation of the N-gain term (see Chapter 84). One general difficulty with calculating the N-gain term is that global estimates are mainly based on N_2-fixation by photoautotrophic diazotrophs in surface waters (~100–200 Tg N/a), consequently underestimating the global N-gain by not considering a large part of the diazotrophic habitat. These measurements of N_2 fixation cannot balance the nitrogen N-loss (~400 Tg N/a) (Karl et al., 2002; Codispoti, 2007) resulting from microbial processes such as anammox (anaerobic oxidation of ammonium with nitrite to N_2 (Dalsgaard et al., 2003; Kuypers et al., 2003)) as well as denitrification (the 4-step reduction of nitrate to N_2 (Falkowski 1997; Codispoti 2007)).

Over the past years, molecular techniques have led to the discovery of a variety of previously unrecognized diazotrophs in oceanic niches previously not regarded important for N_2-fixation (Hamersley et al., 2011). Those diazotrophs have been demonstrated to express their nitrogenase (*nif*) genes and fix N_2, and might therefore significantly contribute to oceanic N_2-fixation (Löscher et al., 2014; see Chapter 84); however, they have previously not been considered for the oceanic N-budget. The discovery of N_2-fixation in mesopelagic waters in the Pacific (Fernandez et al., 2011; Farnelid et al., 2011; Turk et al., 2011) and the possibility of significant involvement of heterotrophic bacteria in global marine N_2-fixation reinforce the idea that a large term of global marine N input by N_2-fixation is clearly underestimated, mainly by not considering niches other than the oligotrophic ocean surface (e.g., OMZs).

In addition, an experimental bias regarding N_2-fixation rate measurements has been identified (Mohr et al., 2010). Those measurements account for ~90% of the geochemically derived estimates (Falkowski, 1997); thus, obtaining correct rates is highly important when estimating the N input. Classically, direct measurements of N_2-fixation rates are obtained either using the $^{15}N_2$-tracer addition method

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(Montoya et al., 1996) or the acetylene reduction assay (ARA) (Capone, 1993).

Comparing N_2-fixation rates using those two methods already showed a potential discrepancy (Mulholland, 2007), which we now know is most likely due to the incomplete dissolution of the $^{15}N_2$ gas bubble, which is directly injected into a seawater sample (Montoya et al., 1996). In order to improve the quality of the direct N_2-fixation rate measurements, a novel method was established involving addition of $^{15}N_2$-enriched seawater, which assured a well-defined and constant $^{15}N_2$ enrichment of the dissolved N_2 gas at the beginning of the incubations. This method has subsequently been tested and validated in the environment (Großkopf et al., 2012).

87.2 COMPARING N_2-FIXATION RATES CALCULATED USING THE TWO METHODS

In order to test the impact of this method in the environment, the magnitude of the underestimation of N_2-fixation rates was investigated in an open-ocean setting. During two research cruises to the Atlantic Ocean (AO) between 25°N and 45°S, investigators compared the established $^{15}N_2$ tracer method for measuring N_2 fixation (bubble-addition method (Montoya et al., 1996)) with the recently developed method (Mohr et al., 2010) in which the $^{15}N_2$ tracer is added as a dissolved gas (the dissolution method). At each station, parallel incubations were conducted with the dissolution and the

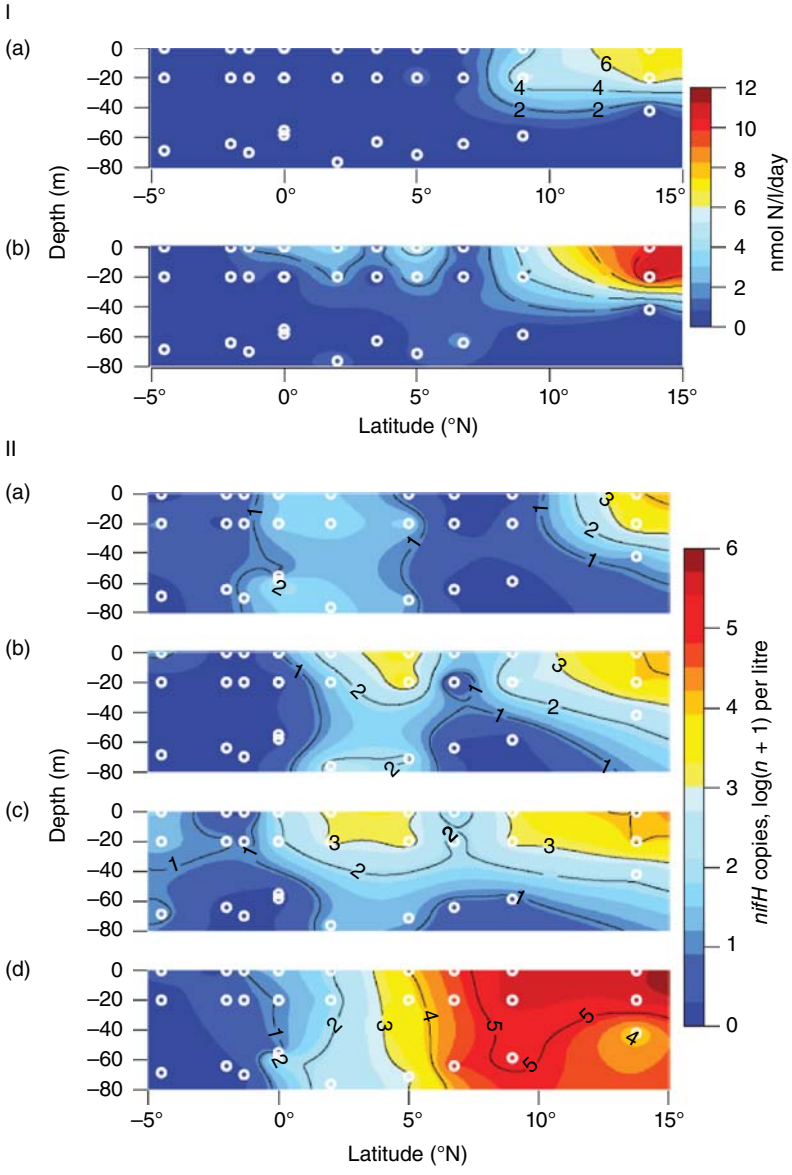

Figure 87.1 I Mixed-layer inventory of N_2-fixation rates in the tropical and equatorial Atlantic Ocean. a, N_2-fixation rates measured with the bubble-addition method. b, N_2-fixation rates measured with the dissolution method. II Relative abundance of various phylotypes of diazotrophic bacteria from the same stations as the N_2-fixation rate measurements, estimated with TaqMan *nifH* gene assays. a, Unicellular cyanobacteria (Group A, Group B and Group C cyanobacteria). b, Diatom-associated heterocystous cyanobacteria. c, Diazotrophic γ-proteobacteria. d, *Trichodesmium*. There was no DNA sampling at the 3.5°N station. Note that the *nifH* gene copies (n) per liter of diatom-associated heterocystous cyanobacteria (such as *Richelia*) detect all cells of the symbionts, not just the heterocysts in which N_2-fixation is actively taking place. This figure was originally published in Großkopf et al. (2012).

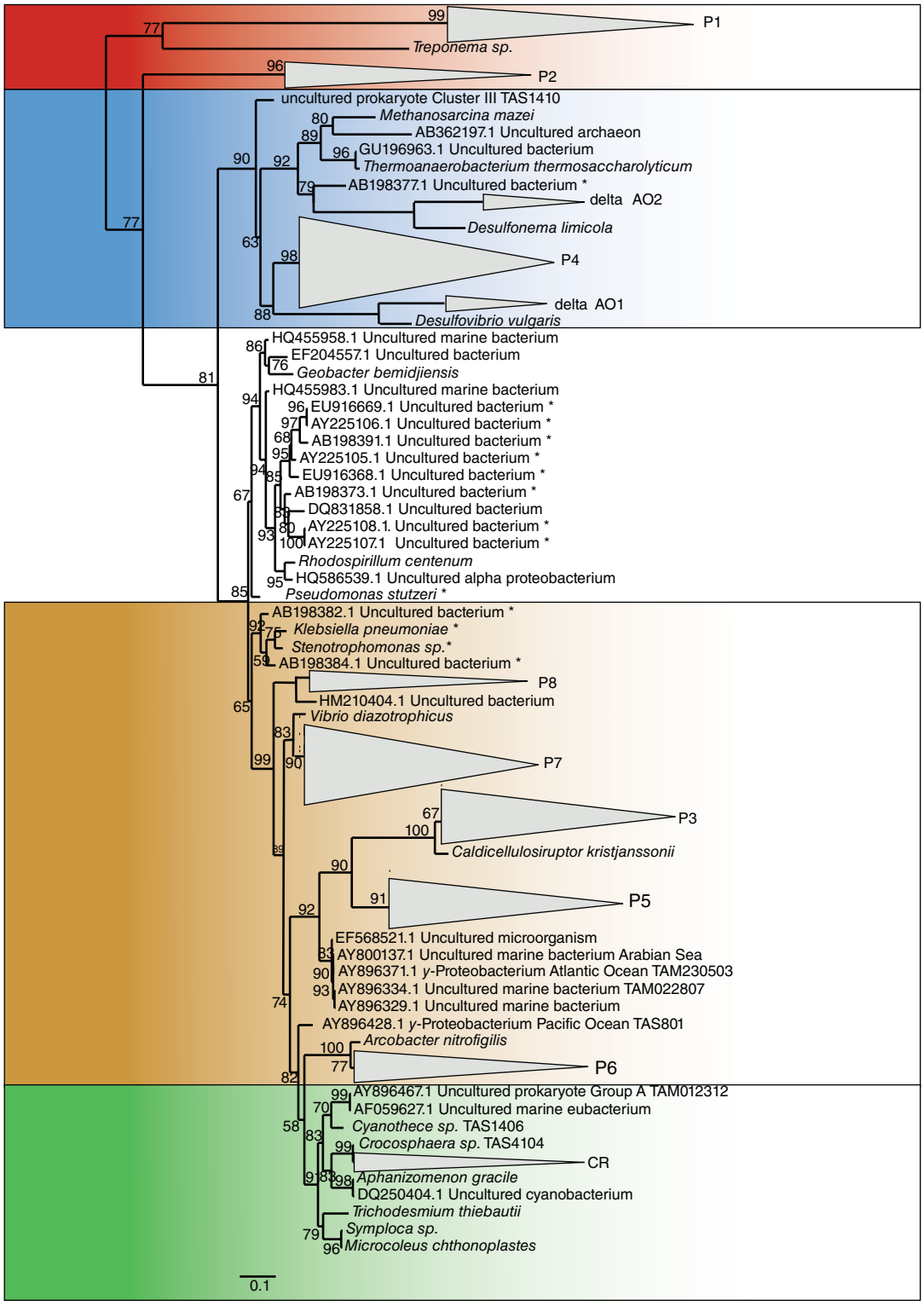

Figure 87.2 Phylogenetic tree based on the analysis of ~600 *nifH* gene and transcript sequences retrieved in Löscher et al. (2014). The newly identified clusters are indicated with grey triangles. Cyanobacterial sequences are highlighted in green, proteobacterial sequences are highlighted in brown, Cluster III sequences as defined by Zehr et al. (2000) are shown in blue, and two novel deep branching clusters are highlighted red. Bootstrapped values (%) above 50, out of 100, are shown on branches. The scale bar represents 10% estimated sequence divergence. Sequences marked with an asterisk indicate likely contaminated PCR products previously reported by Turk et al. (2011); the novel clusters are mostly distant from those sequences.

bubble-addition methods, using dual labeling with $^{13}CO_3$ (to check for differences in biological activity) and $^{15}N_2$ gas in the same incubation bottles to measure dissolved inorganic carbon (DIC) assimilation and N_2-fixation simultaneously with both methods. There was a large difference and poor overall correlation between the N_2-fixation rates calculated using the two methods.

Average depth-integrated rates of N_2-fixation over the whole AO (25°N to 45°S) differed by a factor of 1.7 for the dissolution and bubble-addition methods, respectively, with a geographical pattern emerging among the differences in the rates measured with the two methods (Großkopf et al., 2012).

Moreover, a region previously not considered important for N_2-fixation (Moore et al., 2009) was found in the equatorial AO (4.5°N to 5°S). Here, depth-integrated N_2-fixation rates were on average 570 % higher with the dissolution method than with the bubble-addition method (Fig. 87.1).

87.3 PRESENCE, DISTRIBUTION, AND EXPRESSION OF THE KEY FUNCTIONAL GENE FOR NITROGENASE REDUCTASE (*nifH*) IN NOVEL HABITATS

Comparing the detected N_2-fixation with the distribution of the nitrogenase reductase (*nifH*) gene (see Chapters 3, 8, 20, 77), highest N_2-fixation rates were found inside a *Trichodesmium* bloom measured with the dissolution and bubble-addition methods, respectively. Throughout the tropical North Atlantic (5–15°N), *Trichodesmium* was dominant, and the underestimation of N_2-fixation rates by the bubble-addition method was less severe but still significant. However, the largest underestimation (570%) with the bubble-addition method was detected in the equatorial Atlantic (4.5°N to 5°S), an area dominated by diazotrophs other than *Trichodesmium*. This indicates that the magnitude of underestimation in N_2-fixation rates measured with the bubble-addition method relative to the dissolution method correlated with the composition of the diazotrophic community. A growing knowledge of the phylogenetic diversity of diazotrophs adds up to those results (Turk et al., 2011; Farnelid et al., 2011; Fernandez et al., 2011; Löscher et al., 2014).

Our phylogenetic studies on *nifH* presence and distribution in the eastern tropical North Atlantic revealed the presence of several *nifH* clusters, e.g. filamentous and unicellular cyanobacteria, as well as γ-proteobacteria belonging to the AO cluster (Langlois et al., 2005; Zehr et al., 2003). Moreover, two novel clusters could be identified (delta AO1, delta AO2; (Löscher, Joshi, et al., unpublished)) (Fig. 87.2), both closely related to heterotrophic organisms belonging to the δ proteobacteria. Although both novel clusters were present in low abundances compared to previously described diazotrophs, their potential to fix N_2 awaits closer investigations.

In the OMZ off Peru, seven novel potentially heterotrophic *nifH* clusters (P1–P7) were identified, which have not been described before. Clusters P1 and P2 (Fig. 87.2) are deep branching compared to all previously described clusters. In addition to those newly identified clusters, sequences of two already known clusters (P8 and *Crocosphaera*, (Zehr et al., 2003)) were detected. Cluster-specific TaqMan probes were designed according to the present sequences and gene abundances were determined along vertical and horizontal gradients using quantitative (q)PCR. Clusters P1 and P4 showed significant abundances up to 10^6 copies per liter.

Moreover, the different clusters were distributed along certain patterns, e.g. the P1 cluster appears to be associated with deeper waters (100–300 m), whereas the P2 and P3 clusters show higher abundances in surface waters. Contrary to the general belief that diazotrophs thrive mainly in N-depleted waters, the P1, P4, and P8 clusters were present at high nitrate concentrations (up to ∼40 μmM); *Crocosphaera*, commonly regarded as one of the most important diazotroph in the ocean (Church et al., 2005; Zehr, 2011), was only present in the more oligotrophic parts of the OMZ off Peru. Active nitrogen fixation and the presence of *nifH* genes measured along 10°S point toward a key role of the newly identified clusters for nitrogen fixation in this area.

On the 10°S transect, a broad peak of nitrogen fixation extending into the oxygen minimum zone could be observed. The N_2-fixation activity could be stimulated by the addition of glucose and further by the addition of oxygenated water and glucose. This suggests that heterotrophic diazotrophs thrive within the upper portion of the oxygen minimum zone that seems to be limited by the availability of reduced carbon compounds. Further south of 10°S the N_2-fixation rates increased and showed higher activity at the surface than at depth, which is consistent with the very low N:P ratios in that area. The highest rates of 24.8 +/− 8.4 nmol/day/l were measured at the sulphidic station on the Peruvian shelf at 13.75°S in surface waters. The whole station's water column nitrogen fixation exceeded 1 mmol N/day/m^2, comparable to rates reported from major *Trichodesmium* blooms.

87.4 DISCUSSION AND CONCLUSION

Using published measurements of N_2-fixation rates obtained with the bubble-addition method, a global total of 103 ± 8 Tg N/a (Großkopf et al., 2012) has been calculated. In contrast, our average N_2-fixation rates measured with the dissolution method rises, in case it applies equally to all ocean basins, to 177 ± 8 Tg/N/a (Großkopf et al., 2012).

This difference can first be ascribed to the gap between the novel and the classical methods; however, the presence of

a large number of *nifH* phylotypes other than *Trichodesmium* in the Atlantic and Pacific oceans represents an additional factor leading to the underestimation of N_2-fixation in the ocean.

We identified two major reasons for the underestimation of marine N_2-fixation: (i) a methodological bias, which could be solved by the development and application of a novel method; and (ii) the detection of novel diazotrophic clusters of potentially heterotrophic diazotrophs in the Atlantic and Pacific OMZs. The activity of diazotrophs below the euphotic zone has currently not been considered in estimates of global N_2-fixation rates – neither from biogeochemical models, nor from geochemical tracer studies or direct measurements – and, combined with the underestimation from the classical method, might have been underestimated significantly.

Together, our results demonstrate that the marine N budget might be balanced when calculated using more realistic rate measurements and considering the full habitat of diazotrophs, which exceeds the oligotrophic ocean surface by far.

ACKNOWLEDGMENTS

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Section 16

Diazotrophic Plant Growth Promoting Rhizobacteria and Nonlegumes

Chapter 88

One Hundred Years Discovery of Nitrogen-Fixing Rhizobacteria

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88.1 DISCOVERY OF SYMBIOTIC AND NON-SYMBIOTIC NITROGEN FIXATION

What is the source of plant nitrogen? This topic was reviewed by Prof. P. W. Wilson in 1957, to commemorate the centennial of the Rothamsted experiments on nitrogen fixation, and by Prof. P.S. Nutman (FRS) in the "Centenary lecture," which he contributed at a meeting of the Royal Society (London) (Fig. 88.1a) in 1987, to commemorate the discovery of nitrogen fixation by nodulated legumes by the German scientists Hellriegel and Wilfarth (Wilson, 1957; Nutman, 1987). Both authors acknowledged the work of the two Germans, but they also recalled former contributions by the French scientists Boussingault, Ville, and Jodin, and the English scientists Lawes, Gilbert, and Plugh (from Rothamsted) who tried to demonstrate that plants could use nitrogen gas from the atmosphere, an hypothesis strongly refuted by their contemporaries, in particular, by the great plant nutritionist Liebig who was totally against this possibility. Beijerinck isolated a microorganism from nodules in 1988, named *Bacillus radicicola* (*Rhizobium leguminosarum*), responsible for the nitrogen fixation process in symbiosis with the host plant, but unable to fix nitrogen in the free-living state (Fisher and Newton, 2004; Elmerich, 2007).

Another author is Pierre Eugène Marcellin Berthelot (Fig. 88.1b) who discovered nitrogen fixation in clay soils (Doremus, 1907), although Winogradsky (1949) criticized his data. Berthelot (1827–1907) was a professor of organic chemistry at the Collège de France, in Paris. He was extremely popular and famous in his time as a scientist and a politician. He became Perpetual Secretary of the Academy of Sciences (succeeding Louis Pasteur in 1889), as well as a senator and minister (Langlois-Berthelot, 2000). He was the first scientist to perform synthesis of organic compounds such as acetylene and benzene. In 1883 he founded a laboratory in Meudon, near Paris, which included glasshouses and field plots. It was there that he first discovered chemical nitrogen fixation by lightning, incubating samples on top of a 28 m high tower constructed for that purpose. He performed an incredible number of experiments using soils with and without plants (common vetch, wheat, etc.). By comparing the nitrogen gain of sterilized and non-sterilized soil (without plants) he concluded that microscopic living organisms in the soil were responsible for the fixation of atmospheric nitrogen (Berthelot, 1885). Berthelot (1893), in conjunction with his colleague Guignard, tried to isolate the responsible microbe(s) from the soil, from which they reported the isolation of several unidentified bacterial isolates that "potentially" could be involved in the nitrogen fixation process.

It was Serge Winogradsky (Fig. 88.1c) who, in St-Petersburg, isolated the first bacterial strain capable of nitrogen fixation in the free-living state. Serge Winogradsky (1856–1953) was born in Kiev. He studied both music and plant physiology, and finally turned his interest to bacteriology. He had a very productive scientific career,

*Retired member.

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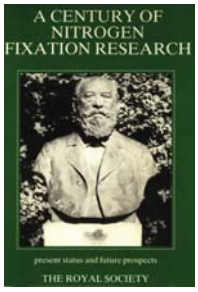

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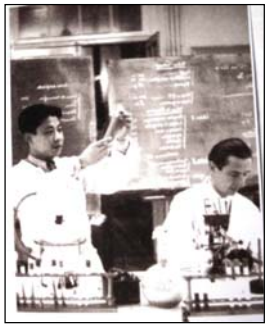
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Figure 88.1 Collection of portraits. (a) Book cover showing Hellriedel bust; (b) Marcellin Berthelot in his laboratory; (c) Serge Winogradsky; (d) Yao-Tseng Tchan at a meeting in Florence, in 1990; (e) Tchan and Jacques Pochon teaching at Institut Pasteur in 1949; (f) Johanna Döbereiner in 1987; (g) Fraser Bergersen, in 1995; (h) cover of the F. Bergersen's book; (i) Yvon Dommergues; (j) Christina Kennedy, in 1996; (k) Robert Burris, in 1988; (l) Yaacov Okon, in 1982; (m) Anton Hartmann, in 1985; (n) Jacques Balandreau, in 1982.

studying bacteria of the sulfur cycle, iron cycle, nitrification, cellulose degradation, and nitrogen fixation, and established the principle of lithotrophy. At the age of 66, in 1922, he accepted the invitation of Emile Roux, to become a staff member of the Institut Pasteur and founded a laboratory, outside of Paris, at the Brie-Comte-Robert where he continued to work, with the help of his daughter Hélène (Courrier, 1957; Dworkin, 2012). In the last years of his life Winogradsky prepared a volume containing commented copies of his main publications as well as his memories and views on soil microbiology and ecology (Winogradsky, 1949).

Winogradsky (1893, 1894) reported the isolation of a nitrogen-fixing culture and showed that it contained an anaerobic spore-forming bacterium. In a paper published in the Archives of St-Petersburg Imperial Institute in 1895, Winogradsky obtained a pure culture resembling *Clostridium,* a genus described by Prazmowski in 1880. It differed from *C. butyricum* by its fermentation products and he

proposed the name *Clostridium pastorianum* (now *pasteurianum*) (see Winogradsky, 1949). Further characterization of the bacterium was published in 1902 (Winogradsky, 1902). A dozen of other *Clostridium* species were confirmed to fix nitrogen in 1949 (Rosenblum and Wilson, 1949).

In 1901, Beijerinck isolated *Azotobacter.* The nonsymbiotic nitrogen fixation era was born.

88.2 *Azotobacter* AND OTHER RELATED NITROGEN-FIXING BACTERIA

Considering the existing knowledge and techniques, progress in the isolation and proper identification of new species of nitrogen-fixing bacteria, as well as the demonstration of their nitrogen fixating ability, remained tedious until the early 1970s (Balandreau, 1983) (Table 88.1). New isolates of free-living, gram-negative, aerobic nitrogen-fixing rods were often considered to be part of the azotobacters and initially named as an *Azotobacter* species (Table 88.1). Thus, the family *Azotobacteraceae* now comprising *Azotobacter* and *Azomonas* genera (De Smedt et al., 1980; Tchan and New, 1984) was reported to also include *Beijerinckia* (Jensen, 1954) and *Derxia,* and even *Azospirillum* (see Elmerich, 1984a).

Martinus Willem Beijerinck (1851–1931) was born in Amsterdam. He was a pioneer in plant virology with his work on tobacco mosaic virus. He isolated a number of bacterial and yeast species working at the Netherlands Yeast and Alcohol Manufactory in Delft. He became a professor at the Delft Polytechnic School in 1895, and remained so until his retirement in 1921 (Chung and Ferris, 1996). In 1901, he isolated two strains of free-living nitrogen-fixing bacteria from soil and water, which he called *Azotobacter chroococcum* and *Azotobacter agilis* (Table 88.1) (see Soriano, 1939). *A. chroococcum* strains produced a brownish pigment and formed cysts. *A. agilis,* producing a greenish pigment with no cysts, was for some time believed to be uniquely found in the Delft canal (Soriano, 1939). Two other *Azotobacter* species, designated *vinelandii* and *beijerinckii*, were obtained by Lipman, during the period 1903–1904 in the United States (Soriano, 1939; Jensen, 1954; see Chapters 7, 9). Beijerinck isolated another strain, in 1922, named *Azotobacter spirillum*, classified later as *Azospirillum lipoferum* (see *Azospirillum* section). *Azotobacter indica*

Table 88.1 Main Free-Living Nitrogen-Fixing Genera or Species Known in 1966

Year of Isolation/or Discovery of N Fixation	Genus or Species Initial Name	Final or Intermediary Name	Source or Reference
1893–1895	*Clostridium pastorianum*	*Clostridium pasteurianum*	Winogradsky (1902)
1901	*Azotobacter chroococcum; A agilis*	—	Beijereinck, see: Soriano (1939)
1903–1904	*Azotobacter vinelabndii, A. beijerinckii*	—	Lipman See: Soriano (1939)
1922–1925	*Azotobacter spirillum; Spirillum lipoferum*	*Azospirillum lipoferum; A. brasilense* (1978)	Beijerinck See: Becking (1963); Tarrand et al., (1978)
1928	*Aerobacter aerogenes*	*Klebsiella*	Skinner See: Stewart, 1967
1928*	*Nostoc; Anabaena*	—	Drewes See: Stewart, 1967
1938	*Azomnas agilis*	—	Winogradsky, (1938)
1939	*Azotobacter indica*	*Beijerinckia indica* (1950)	Starkey and De (1939); Derx (1950)
1949†	*Rhodospirillum rubrum*	—	Kamen and Gest (1949)
1950	*Beijerinckia*	—	Derx (1950)
1958	*Bacillus polymyxa*	*Paenibacillus* (1999)	Hino & Wilson (1958); Achouak et al., (1999)
1958	*Beijerinckia fluminensis*	*B. doebereinerae* (2009)	Döbereiner & Ruschel (1958); Oggerin et al., (2009)
1960	*Derxia*	—	Jensen et al., (1960)
1965	*Klebsiella*	—	Mahl et al., (1965)
1966	*Azotobacter paspalum*	—	Döbereiner (1966)

*It was not proved for sure that blue green algae could fix nitrogen before 1928 due to the difficulty in obtaining pure culture.

†Nitrogen fixation in *R. rubrum* was not known before 1949; it was demonstrated by using $^{15}N_2$.

was isolated in 1939 (Starkey and De, 1939) and classified later as *Beijerinckia indica* (Derx, 1950) (Table 88.1).

In the Brie-Comte-Robert laboratory, Winogradsky developed several methods for studying the dynamics of *Azotobacter* populations in the natural environment and he is considered to be the first microbial ecologist (Courrier, 1957; Dworkin, 2012). He developed the use of silica gel plates that were devoid of combined nitrogen or sifted soil, and to which mannitol or other carbon sources were added. Applying a range of different physiological conditions resulted in changes in the concentrations of samples of *Azotobacter.* In particular, the addition of a carbon source led to a great increase of *Azotobacter* colonies. He proposed that the number of *Azotobacter* colonies present in soil were correlated to the nitrogen-fixation potential of the soil (Winogradsky, 1926; 1932; 1949).

Winogradsky (1938) re-examined the classification of azotobacters. The initial *A. agilis* strain of Beijerinck was lost. He succeeded in isolating strains corresponding to *A. agilis* from water in France. Subsequently, by comparing a number of *Azotobacter* strains from different collections to the water isolate, he concluded that *A. agilis* corresponded to a different genus that he called *Azomonas.*

Jacques Pochon (1907–1978), a medical doctor working at the Pasteur Institute laboratories in Garches, near Paris, was interested in pursuing studies in soil microbiology, in particular nitrogen fixation and cellulose decomposition. He recruited Tchan Yao-Tseng (Fig. 88.1d) in 1945. Tchan (1918–2013) came to France in 1937 to study agriculture. Maurice Javillier (1875–1955) was his professor. He obtained a Doctorate Thesis at the Sorbonne, in Paris, in microbial ecology. Familiar with the work and the techniques of Winogradsky he was eager to work with him, but Winogradsky, who was already advanced in age, did not welcome students and suggested to Tchan to cooperate with his daughter Hélène (1890–1957) at the Institut Pasteur campus in Paris. Tchan chose to integrate into the laboratory of Jacques Pochon, first in Garches, and then in Paris (Tchan, personal communication) resulting in a fruitful 4 years of collaboration. Pochon and Tchan each contributed to the teaching of the "*Grand Cours de Microbiologie*" (Fig. 88.1e); they published a number of papers on *Azotobacter* and related topics in the *Annales de l'Institut Pasteur,* as well as the *Manual of Soil Microbiology* (Pochon and Tchan, 1948).

Subsequently, Tchan accepted a position as Senior Microbiologist of the Linnaean Society of New South Wales in 1950 and migrated to Australia. He later established the Chair of the Department of Microbiology at the University of Sydney for the students of Science and Agriculture. He continued his work, in Australia on the taxonomy, physiology, and biochemistry of *Azotobacter*, *Azomonas*, *Beijerinckia*, and also on cyanobacteria (e.g., Tchan, 1952, 1953; New and Tchan, 1982; Tchan and New, 1984, Tchan et al., 1980, 1983). Tchan collaborated with the famous Danish microbiologist Hans Laurits Jensen (Tchan et al., 1962). HL Jensen (1898–1977) described *Derxia*, another acid-tolerant strain (Jensen et al., 1960), named in honor of the Dutch microbiologist HG Derx (1894–1953).

Emeritus Professor Tchan continued to work on *Azospirillum* and I was fortunate to work with him for a short time in Sydney (Zeman et al., 1992). To quote in his communication to me: "*I started my research in the Pasteur Institute and finished it with the collaboration of a fellow Pasteurian.*"

By 1952, Pochon created a theoretical and practical "Soil microbiology course" in the Pasteur Institute, which attracted students from France and from many other parts of the world. Johanna Döbereiner (Fig. 88.1f) followed that course in 1956. She discovered two other species of azotobacter-related bacteria: *Beijerinckia fluminensis* (Döbereiner and Ruschel, 1958) and *Azotobacter paspali* (Döbereiner, 1966) (Table 88.1).

88.3 CONTRIBUTION OF *Azotobacter* TO SOIL FERTILITY

For many years *C. pasteurianum* and *Azotobacter* were considered as the main non-symbiotic nitrogen-fixing bacteria in soil (Winogradsky, 1949; Jensen, 1954; Chang and Knowles, 1965). Indeed, proliferation of bacteria in the root zone of plants was noted very early; this is the rhizosphere effect described by Hiltner in 1904 (Starkey, 1958; Hartmann et al., 2008). Most of the bacteria isolated from plants were considered as saprophytes. *Azotobacter*, which was not found in higher concentration in the root zone than in the soil, was not considered a rhizospheric bacterium (Starkey, 1958; Döbereiner, 1974).

Bacterization experiments (1927–1960) were initiated in the Soviet Union because Russian soils contained large numbers of *Azotobacter*. By 1958, about 10 million ha were treated with the fertilizer azotobacterin, a preparation of *A. Chroococcum,* and with phosphobacterin, containing *Bacillus megaterium* (Macura, 1966; Brown, 1974). The *Bacillus* was used for organic phosphate mineralization. Results of bacterization generated controversy. Up to 10% yield increase with cereals was reported and even up to 50% with some vegetables (Brown, 1974; Mishustin, 1970). Mischustin (1970) in an analytical report noted that 10% increase might be considered "*within the limit of field experiment error.*"

Research programs were conducted in other parts of the world – California, Australia, and England (in particular in Rothamsted) – to understand how these bacterial fertilizers could replace mineral fertilizers (Brown, 1974). Delwiche and Wijler (1956) performed experiments using $^{15}N_2$. In particular, they confirmed nitrogen fixation by soil, with dramatic increase when a substrate such as glucose was added to the soil. However, they found little increase when

the soil was amended with straw or when it was inoculated with *Azotobacter* (in contrast to Chang and Knowles (1965) who reported increases after *Azotobacter* inoculation).

Many studies dealt with occurrence of *Azotobacter* in soil and its survival after inoculation. It appeared that *Azotobacter* concentration in soil was rather low (some soils which were too acidic did not contain any), that no effective colonization of the rhizoplane was found, and that the survival of the bacteria after inoculation was poor (Döbereiner, 1974). Meiklejohn (1965) concluded from the experiments she performed on Broadbalk field (Rothamsted) that "*Perhaps,* Azotobacter *indicates conditions for fixation but the fixation is mainly by other species*" and she suggested that *C. pasteurianum* instead could be effective. Chang and Knowles (1965), in Canada, reached a similar conclusion. These authors identified *C. pasteurianum* from most of the studied soil samples whereas *Azotobacter* was rarely present. By using $^{15}N_2$ they detected nitrogen fixation in anaerobic conditions with an increase in anaerobes and aerobes populations. They concluded that, in addition to *C. pasteurianum*, nitrogen fixers other than *Azotobacter* were present in Canadian soil.

Many authors noted that the inoculation of *Azotobacter* resulted in the modification of plant morphology (Brown, 1974). For example, Patel (1969) showed spectacular increases in plant growth after the inoculation of wheat seedlings; Dénarié and Blachère (1966) noted growth stimulation on several plants (potato, tomato, ray-grass), but positive effects were also found with *Arthrobacter* and *Xanthomonas*; Rovira (1963) equally reported stimulation of plant growth by *Azotobacter* and also by *Clostridrium*. Bacterization using non-nitrogen-fixing strains (*Bacillus*, *Pseudomonas*) also resulted in plant growth promotion (PGP) (Brown, 1974). Production of vitamins, amino acids, phytohormones, and antifungal metabolites by soil- and plant-associated microorganisms was established (Vancura, 1961; Rivière, 1963; Macura, 1966; Brown, 1974, Rovira, 1991). It was concluded that production of bacterial metabolites resulted in promoting plant growth or protection of the plant from diseases. Therefore, the PGP effect resulting from *Azotobacter* bacterization was not linked to nitrogen fixation.

An exception is the case of the specific association between *Azotobacter paspali* and the grass *Paspalum notatum* cv. Batatais (Döbereiner et al., 1972). Effective nitrogen fixation by this association was demonstrated by the ^{15}N isotope dilution method (Boddey et al., 1983).

88.4 *Azotobacteraceae* AND THE CLASSIFICATION IN *Proteobacteria*

Azotobacter distribution was found to be limited to neutral soils. The acid-tolerant *A. indica* species with a smaller cell size was isolated from tropical soil and reclassified as *Beijerinckia indica* by Derx (1950). It was emphasized that *Beijerinckia,* which predominates in tropical soil, could be of agricultural importance (Becking, 1961; Döbereiner, 1961; Dommergues, 1963; Dommergues and Mutaftschiev, 1965; Döbereiner, 1974). In particular, Döbereiner (1961) reported the presence of *Beijerinckia* in the rhizosphere of sugarcane. Both of the *Azotobacter* and *Beijerinckia* genera differed in major properties; for example, *Azotobacter* could form cysts in old cultures and fix nitrogen with vanadium instead of molybdenum, while *Beijerinckia* could not (Becking, 1962). However, based on phenotypic features the differentiation between *Azotobacter, Beijerinckia* and *Derxia* remained difficult.

Jozef de Ley (1924–1997) played a pioneering role in the classification of nitrogen-fixing bacteria. He founded the "Laboratory for Microbiology" of the Ghent University, Belgium, and with his team contributed to the taxonomy and phylogeny of many bacterial groups. Development of molecular techniques based on DNA base composition and DNA–DNA hybridization enabled the confirmation that *Azotobacter*, *Azomonas, Beijerinckia,* and *Derxia* belonged to different genera (De Ley and Park, 1966; de Ley, 1968). With his team he then performed an extensive analysis of hybridization of DNA with ribosomal RNA. This technique enabled to group gram-negative bacteria within four "rRNA superfamilies" and showed that *Beijerinckia*, *Azotobacter,* and *Derxia* belonged to different RNA superfamilies (De Smedt et al., 1980; de Vos et al., 1985). Tchan et al. (1980, 1983) used immunoelectrophoresis to better differentiate *Azotobacter* and *Azomonas* genera.

The classification of rRNA superfamilies was replaced by that proposed by Carl Woese (1922–2012) and collaborators who first used RNA oligonucleotide catalogs, and then the sequencing of 16S rDNA, classifying gram-negative bacteria (purple bacteria) into alpha, beta, gamma, and delta subgroups (Woese, 1987). The term *Proteobacteria*, currently used, was proposed in 1988 (Stackebrandt et al., 1988). Thus, *Azotobacter* is a member of the gamma subgroup of *Proteobacteria* close to *Pseudomonas, Beijerinckia* is an alpha *Proteobacteria* close to *Rhizobiales,* and *Derxia* belongs to the beta subgroup.

88.5 *Azospirillum* HISTORY

Johanna Döbereiner and her team discovered bacteria of the genus *Azospirillum* in Brazil in the early 1970s (Döbereiner and Pedrosa, 1987; Baldani and Baldani, 2005). Johanna Döbereiner (1924–2000) was born in Czechoslovakia and studied agronomy at the University of Munich. She emigrated to Brazil in 1951, where she established a soil microbiology laboratory in Seropédica, 42 km from Rio de Janeiro (Figures 88.1f and 88.2a). Her discovery of several new genera of root-associated diazotrophs that benefit the growth of non-leguminous plants stimulated research of

Figure 88.2 Pictures from Brazil and Asia. (a) Johanna Döbereiner in her laboratory in 1987; (b, c) Vera and Jose Ivo Baldani, in 1995; (d) Fabio Pedrosa (right) and his group, in Curitiba, in 1987; (e) Christina Neves and Robert Boddey, in 1995; (f) Ben Bolhool (1940–1991), Chong Biao You and Kauser Malik, at a meeting in Philippines (IRRI) in 1985; (g) Mercedes Umali-Garcia in 1982; (h) CB You and Lin Min, in Beijing, in 1994; (i) Iowa Watanabe at IRRI, in 1985; (j) JK Ladha, in 1985; (k) Kiwamu Minamisawa, in Kyoto in 2012.

non-symbiotic nitrogen fixation all over the world (reviewed by Baldani and Baldani, 2005). Avílio Franco and Robert Boddey organized an international symposium in Angra dos Reis (Rio de Janeiro, Brazil) in 1995, to commemorate Johanna's 71st birthday (Fig. 88.2e). Many of her friends and former collaborators attended the meeting. The proceedings were published as a special issue of *Soil Biology and Biochemistry* (Vol. 29, No 5/6, 1997). Fraser Bergersen (Fig. 88.1g) (1929–2011), Yvon Dommergues (Fig. 88.1i) (1922–2009), and Christina Kennedy (Fig. 88.1j) (1945–2008) contributed to the meeting.

The first record of nitrogen fixation by a spirillum-like bacterium, named *Azotobacter spirillum*, then *Spirillum lipoferum,* was in the period 1922–1925 (Becking, 1963), although the strain could not fix nitrogen in pure culture (Döbereiner, 1988). Jan-Hendrix Becking (1924–2009), a Dutch microbiologist, isolated a strain in Africa that he probably considered identical to Beijerinck's *S. lipoferum* strain (Becking, 1963). He showed nitrogen fixation in pure culture, using $^{15}N_2$, but could not conclude on the taxonomic status of the strain (Becking, 1963).

The strains isolated in Brazil were first named *Spirillum lipoferum* Beijerinck. An ecological survey showed that *S. lipoferum* was present in different parts of Brazil, United States, and Africa, and it was isolated from soil, roots of forage grass (*Digitaria, Panicum, Pennisetum*), and cereal

Table 88.2 *Azospirillum* and Other Nitrogen-Fixing Soil and Plant-Associated/Endophytic Bacteria (1973–2001)

Year of Discovery or First Publication	Genus or Species [Initial] Name	Country/Plant Rhizosphere	Final or Intermediary Name	Reference
1973–1974	*Spirillum lipoferum*	Brazil; *Digitaria decumbens*	*Azospirillum* (1978) A. *brasilense; A. lipoferum*	Döbereiner & Day, (1976) Tarrand et al., (1978)
1980	*Campylobacter*	Canada; *Spartina alterniflora*	—	McClung & Patriquin (1980)
1981	*Klebsiella planticola*	—	—	Bagley et al., (1981)
1982	*Alcaligenes faecalis*	China; rice	*Pseudomonas stutzeri* (1999)	You & Qu (1982) Vermeiren et al., (1999)
1982	*Pseudomonas paucimobilis*	France; rice	*Flavobacterium paucimobilis* (1983) *Sphingomonas paucimobilis* (1990)	Bally et al., (1983) See: Chan et al., (1994]
1983	*Azospirillum amazonense*	Brazil	—	Magalhães et al., (1983)
1984	*Bacillus azotofixans*	Brazil	*Paenibacillus azotofixans*	Seldin et al., (1984) Achouak et al., (1999)
1984–1986	*Azospirillum seropedicae*	Brazil; maize	*Herbaspirillum seropedicae*	Baldani et al., (1986) Döbereiner (1992)
1987	*Azospirillum halopraeferans*	Pakistan; Kallar grass	—	Reinhold et al (1987)
1987	*Pseudomonas stutzeri*	Germany; sorghum	—	Krotzky & Werner, (1987)
1988	*Acetobacter nitrocaptans* (1988) *Acetobacter diazotrophicus* (1989)	Brazil; sugarcane	*Gluconacetobacter diazotrophicus* (1998)	Gillis et al., (1989) Döbereiner (1992) Yamada et al., 1997
1989	*Azospirillum irakense*	Iraq; rice	—	Khammas et al., (1989)
1990	*Pseudomonas rubrisubalbicans*	Brazil; sugarcane	*Herbaspirillum rubrisubalbicans*	Döbereiner (1992)
1993	*Azoarcus*BH72 *Azoarcus communis* *Azoarcus indigens*	Pakistan; Kallar grass	—	Reinhold-Hurek et al., (1993)
1993	*Azoarcus* Sp. group C	Pakistan; Kallar grass	*Azovibrio restictus* (2000)	Reinhold-Hurek et al., (1993) Reinhold-Hurek and Hurek (2000)
1993	*Azoarcus* Sp. group D	Pakistan; Kallar Grass	*Azospira orizae*	Reinhold-Hurek et al., (1993) Reinhold-Hurek and Hurek (2000)
1994	*Burkholderia vietnamiensis*	Vietnam; rice	—	Trân et al., (1994)
1997	*Azoarcus* Sp. group E	Pakistan; fungal sclerotia	*Azonexus fungiphilus* (2000)	Reinhold-Hurek and Hurek (2000)
1997–2000	*Burkholderia tropicalis*	Brazil; pineapple, banana	*Burkholderia tropica*	Rothballer et al., (2007)
2000	*Klebsiella pneumoniae* Kp 342	USA; maize	—	Chelius and Triplett (2000)
2001	*Burkholderia* sp.	Mexico; various plants	—	Rothballer et al., (2007)
2001	*Herbaspirillum* sp.	Japan; rice	—	Elbelgaty et al., (2001)
2001	*Azospirillum doebereinerae*	Germany; C4-grass *Miscanthus*	—	Eckert et al., (2001)
2001	*Herbaspirillum frisingense*	Germany and Brazil; forage grasses	—	Kirchhof et al., (2001)
2001	*Gluconacetobacter johannae* *Gluconacetobacter azotocaptans*	Mexico; coffee plant	—	Fuentes-Ramírez et al., (2001)

crops (maize, sorghum, wheat) (Döbereiner et al., 1976). An association was also found with rice in Asia (Watanabe and Roger, 1984).

Based on the nutritional properties of the different isolates, DNA base composition and DNA–DNA hybridization experiments with other bacterial genera, Tarrand et al. (1978) proposed to create a new genus *Azospirillum*, with two species *A. lipoferum* and *A. brasilense*. De Smedt et al. (1980) found that *Azospirillum* was in the same rRNA superfamily as *Beijerinckia*, which is in agreement with its further classification in the alpha subgroup of *Proteobacteria*. Seven species were recognized in 2007: *A. brasilense, A. lipoferum, A. amazonense, A. irakense, A. halopraeferens, A. Largimobile,* and *A. doebereinerae* (Schmid and Hartmann, 2007), but a number of new species have been described in recent years (see Wisniewski-Dyé et al., 2011) (Table 88.2).

Soon after the report of "Spirillum" bacteria promoting growth of Gramineae by J. Döbereiner at the First International Symposium on Nitrogen Fixation, at Pullman, Washington, in (1974), many laboratories in North and South America, Europe, Africa, and Asia started to implement research on this bacterium (see reviews by: Patriquin et al., 1983; Elmerich, 1984a,1984b; Okon, 1985, 1994; Danneberg et al., 1986; Döbereiner and Pedrosa, 1987; Elmerich et al., 1987; Michiels et al., 1989; Bashan and Levanony, 1990; Okon and Labandera-Gonzales, 1994; Costacurta and Vanderleyden, 1995; Steenhoudt and Vanderleyden, 2000; Elmerich and Newton, 2007; Franche et al., 2009; see also Chapter 90).

Azospirillum (as well as many other root-associated diazotrophs) have been isolated after surface sterilization of the plant root. The "intimate" association of *Azospirillum* with the host plant was first referred as "associative symbiosis" because invasion of root cortical cells was noted (Döbereiner and Day, 1976). However, it was argued that the colonization was essentially limited to the surface, but that bacteria could proliferate in damaged tissues and be protected from the sterilizing agent (Umali-Garcia et al., 1980; Hallmann et al., 1997). Use of confocal laser scanning microscopy (Rothballer et al., 2003) helped solve the controversy by noting differences in the localization depending of *Azospirillum* strains. The *A. brasilense* Sp7 bacterial cells were localized preferentially on the root surface, while *A. brasilense* Sp245 was on the surface but also in the intercellular spaces of the root epidermis, and could then be considered as an "endophyte."

Robert Burris (1916–2010) (Fig. 88.1k) had already a brilliant career in the field of the biochemistry of nitrogenase, when he started to work with *Azospirillum*. He also became involved in the study of *Gluconacetobacter* in collaboration with Christina Kennedy (Sevilla et al., 2001). Yaacov Okon (Fig. 88.1l) and Anton Hartmann (Fig. 88.1m) joined Burris' Lab as post-doctorants and contributed to the physiology and biochemistry of the bacterium (Okon et al., 1976, 1977; Ludden et al., 1978; Hartmann et al., 1986, 1987).

In Germany, Walter Klingmüller (Fig. 88.3a), professor of genetics at the University of Bayreuth, organized four *Azospirillum* workshops to discuss the progress of genetics, physiology, and ecology in the field. Proceedings' books were published after each meeting (Klingmüller, 1982, 1983, 1985, 1988). Most of the European laboratories involved in *Azospirillum* research attended these meetings (Fig. 88.3b–e) and, with time, the audience became international and the workshops also included reports on other rhizobacteria. Becking attended the first and third workshops and Johanna Döbereiner attended the second one.

Istvan Fendrik and Maddalena Del Gallo organized the workshop in Hannover, Germany, in 1991 (Fig. 88.3b and e), and the proceedings were published as a special issue of *Symbiosis* (vol. 13, 1992). At this workshop, Johanna Döbereiner reported data on new root-associated diazotrophs, *Herbaspirilum* (isolated in 1986; see Chapter 93) and *Acetobacter diazotrophicus* (*Gluconacetobacter*, isolated in 1988), which could also colonize aerial parts of the host plant (xylem vessels), without disease symptoms and launched the concept of endophytic nitrogen fixers (Döbereiner, 1992) (Table 88.2; see also Section 16 and Chapter 108).

88.6 ABOUT PROGRESS IN ISOLATION, IDENTIFICATION, AND QUANTIFICATION

Johanna Döbereiner (1988) stressed the importance of the use of appropriate selective media. In particular, the success in the isolation of microaerobic nitrogen fixers depended on the use of nitrogen-free semisolid media, which led to the formation of a gradient of decreasing oxygen concentration below the surface. It enabled growth of nitrogen-fixing bacteria, forming a pellicle, at the oxygen concentration compatible with their physiological requirements for synthesis and functioning of the nitrogenase. In their historical review paper, Baldani and Baldani, 2005 (Fig. 88.2b and c) discussed the impact of the method. They recalled that it was Fábio Pedrosa, when working in Seropédica before he founded his group in Curitiba (Fig. 88.2d), who improved in 1975 the recipe of the semi-solid medium, named Nfb; "*N stands for new and Fb for Fábio Pedrosa.*"

Another major issue was to establish that the bacterial isolates could fix nitrogen in the free-living state and to quantify nitrogen fixation in association with the host plant. It may be worth recalling that the Kjeldhal method is dated 1883, and the use of $^{15}N_2$ was adapted to measure N_2-fixation in 1941 (Burris and Miller, 1941). The first crude extract for nitrogenase activity was obtained in 1960 (Carnahan et al., 1960) and the discovery that acetylene was a nitrogenase substrate was dated in 1966 (Dilworth, 1966).

Figure 88.3 *Azospirillum* workshops. (a) Walter Klingmüller and his family, in Bayreuth, Germany, in 1987; (b) delegates at the *Azospirillum* V workshop, in 1991: Paul Kaiser (1932–2010), Wolfgang Zimmer (1958–2002), René Bally and Yao-Yun Liang; (c) Jos Vanderleyden (in 1995); (d) Barbara Reinhold (in 2012); (e) Group picture at the *Azospirillum* V workshop; second row left: Istvan Fendrik and Maddalena del Gallo.

The difficulties and limitations of different techniques to quantify nitrogen fixation with non-legumes are discussed in many review papers (e.g., Hardy et al., 1973; Van Berkum and Bohlool, 1980; Rennie and Rennie, 1983), and Fraser Bergersen (1980) edited a volume of techniques (Fig. 88.1h) that was so useful that it was translated into Chinese.

The acetylene reduction assay (ARA) became a method of choice to assay nitrogen fixation although it should be correlated to quantitative nitrogen fixation with caution (Bergersen, 1970). In particular, ARA performed on excised roots or on plant soil cores was a source of some controversy (Van Berkum and Bohlool, 1980). Many groups implemented research to validate the assay, such as the conversion factor between ARA and nitrogen fixation (theoretically of three) or estimation of diurnal, seasonal, and varietal variations (e.g., Bergersen, 1970; Rinaudo and Dommergues, 1971; Balandreau et al., 1974; Lee and Watanabe, 1977; Hirota et al., 1978; Sano et al., 1981).

Around 1990, rapid development of molecular tools for bacterial identification and phylogenetic studies boosted the research on the ecology of nitrogen fixation. The DNA amplification technique by polymerase chain reaction (PCR), design of oligonucleotides probes specific for ribosomal RNA, or nitrogen fixation genes as well as primers

for DNA fingerprinting enabled not only identification of new bacterial species but also analysis of microbial population (culturable or non-culturable) associated to the plant (Stoltzfus et al., 1997; Schmid and Hartmann, 2007).

88.7 DIVERSITY OF NITROGEN-FIXING ROOT-ASSOCIATED BACTERIA: ROOT COLONIZERS AND ENDOPHYTES

Less than a dozen of "free-living" nitrogen-fixing genera of bacteria were identified by the end of the 1960s, including *Klebsiella*, *Bacillus polymyxa,* and *Rhodospirillum rubrum* (Table 88.1). Nitrogen fixation was also established for a number of blue-green algae (cyanobacteria, which were not considered eubacteria in 1967; see Chapters 84, 86) (Stewart, 1967). Jacques Balandreau (Fig. 88.1n) in his review paper in 1983 cites a few other genera (e.g., *Arthrobacter*, *Achromobacter, Mycobacterium*, and photosynthetic bacteria) not discussed here. The review article of Peter Young (1992) contains a comprehensive list of nitrogen-fixing species that are classified according to the nomenclature proposed by Woese, which is extremely useful to consult.

Research on the bacteria present in the rhizosphere led to the discovery of a number of new species of nitrogen-fixing root-associated bacteria (Table 88.2). Their distribution and frequency differed greatly with the geographical regions and the plant material (Balandreau, 1983; Bally et al., 1983; Rennie, 1980, 1981; Döbereiner and Pedrosa, 1987; Döbereiner, 1992; Elmerich et al., 1992; Baldani and Baldani, 2005; Schmid and Hartmann, 2007; Pedraza, 2008).

In early reports, in addition to *Azotobacter* and *Beijerinckia* and *Azospirillum* (discussed above), *Bacillaceae* (classified later in a new genus *Paenibacillus*) (Seldin et al., 1984; Achouak et al., 1999), *Enterobacteriaceae* (*Klebsiella, Enterobacter*, *Erwinia, Pantoae*) (Ladha et al., 1983; Kleeberger et al., 1983; Gavini et al., 1989), *Campylobacter* (McClung and Patriquin, 1980), and *Pseudomonas*-like bacteria were frequently identified (Table 88.2).

Microbiology of rice was of particular interest for researchers in Asia (Fig. 88.2f–k). Beside cyanobacteria, a number of aerobic and anaerobic heterotophs were found (Watanabe and Roger, 1984; You et al., 1992; Ladha et al., 1997; Malik et al., 1997; Elbeltagy et al., 2001; see Chapter 108).

The existence of nitrogen-fixing *Pseudomonas sensu stricto* was refuted for a long time since a number of early reports corresponded in fact to bacterial species reclassified later as members of the alpha and beta subgroups of *Proteobacteria* (Young, 1992; Chan et al., 1994; Vermeiren et al., 1999; Yan et al., 2008, 2013). Krotzky and Werner (1987) were the first to isolate a nitrogen-fixing *Pseudomonas stutzeri* (see Chapter 10); several other *Pseudomonas* nitrogen-fixing strains, from different geographical origins, have been reported, including *P. stutzeri* A1501 (for a review see, Yan et al., 2013).

P. stutzeri strains (first described as *Alcaligenes faecalis*) isolated from rice root in China, by Chong-Biao You and his team, have been particularly well studied. CB You (1932–1994) (Fig. 88.2h) was a biochemist from the Institute for Application of Atomic Energy of the Chinese Academy of Agricultural Sciences in Beijing. He obtained a PhD in Moscow in 1961 and later worked in California on *Azotobacter* nitrogenase with Barbara Burgess and William Newton. With his team in China he studied many aspects of the ecology, genetics, and biochemistry of bacteria associated to rice (for a review see: You et al., 1992). A variety of nitrogen-fixing strains were found (including *Klebsiella*), but *A. faecalis* (*P. stutzeri*) was predominant (You and Qu, 1982; You et al., 1992). Lin Min (Fig. 88.2h) who continues active research on the molecular biology of *P. stutzeri* is now directing the Chinese group in Beijing (see Chapter 10).

Following J. Döbereiner's publication in 1992, particular interest was focused on the bacterial endophytes. Many new species were isolated from sugarcane, rice, and other plants including coffee, pineapple, and banana (Table 88.2). *Herbaspirillum* and *Gluconacetobacter* biology was particularly well studied and both species were reported to contribute significantly in fixing nitrogen to sugarcane (James, 2000; Baldani and Baldani, 2005; Elmerich and Newton, 2007; Rothballer et al., 2007; Pedroza, 2008).

Azoarcus and several related genera, *Azovibrio*, *Azospira,* and *Azoxenus*, were among the new nitrogen-fixing species isolated in Pakistan from a salt-tolerant plant, Kallar grass, or from fungal resting stages (sclerotia) (Reinhold et al., 1986; Malik et al., 1991; Reinhold-Hurek and Hurek, 1998, 2000; Hurek et al., 2002) (Table 88.2). The best studied strain *Azoarcus* sp. BH72 could invade rice and its complete genome sequence was determined (Krause et al., 2006; see also Chapter 108). Chelius and Triplett (2000), in the United States, reported on a *Klebsiella pneumoniae* strain 342, an endophyte of maize. New species were also reported among *Proteobacteria* of the beta subgroup, for example, *Burkholderia* isolated from rice in Vietnam or from coffee plant in Mexico (Trân Van et al., 1994; Estrada de los Santos et al., 2001; Baldani and Baldani, 2005; Schmid and Hartmann, 2007). *Herbaspirillum* and *Azospirillum* were also isolated as rice endophytes in Japan (Elbeltagy et al., 2001; Xie and Yokota, 2005; Kaneko et al., 2010).

88.8 CONCLUDING REMARKS

For many years, research was focused on symbiotic nitrogen fixation systems because they are the most efficient in deriving nitrogen from the atmosphere. In contrast, the importance of non-symbiotic nitrogen fixation by soil

bacteria or root-associated bacteria was long considered as negligible. *Azospirillum* inoculation resulted in modification of the root morphology and increase in crop yield was consistently noted (Okon and Labandera-Gonzales, 1994; see Chapter 90). The cause of the PGP effect led to controversy. Similarly to what happened in the case of *Azotobacter* bacterization it was concluded that plant growth stimulation resulted essentially from phytohormone production rather than from nitrogen fixation (Okon and Labandera-Gonzales, 1994; Costacurta and Vanderleyden, 1995; Elmerich and Newton, 2007; see Chapter 91). Endophytes offered new perspectives since some of them benefited plant growth through nitrogen fixation (Sevilla et al., 2001; Hurek et al., 2002; Rothballer et al., 2007; Elmerich and Newton, 2007). The research on root-associated and endophytic nitrogen-fixers has never been so active and several chapters of this book cover updated information on various aspects of research on these systems (see Chapters 90, 92, 93, 94, 108, 111).

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Chapter 89

Symbiotic Nitrogen Fixation in Legumes: Perspectives on the Diversity and Evolution of Nodulation by *Rhizobium* and *Burkholderia* Species

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89.1 INTRODUCTION

More than a decade ago, we wrote an update on the *Rhizobium*–legume symbiosis asking the question "what makes this symbiosis so special?" (Hirsch et al., 2001). Since that time, we have learned a great deal more about this important plant–microbe association. However, the story of symbiotic nitrogen fixation in legumes has become more complicated due to the discovery of several Betaproteobacteria, namely species of *Burkholderia* and *Cupriavidus*, which also establish nitrogen-fixing nodules on legumes. In this chapter, we expand our analysis of the *Rhizobium*–legume symbiosis to include the betarhizobia, specifically *Burkholderia*, and their relevance to this well-studied, beneficial interaction between bacterium and plant.

In particular, we examine the concept of host range, biogeography, as well as evolutionary history with respect to nodulation by *Burkholderia* and *Rhizobium* (see also Chapter 17). Phylogenetic analyses strongly indicate that both genera acquired the nodulation genes at approximately the same time as the legumes were evolving (Bontemps et al., 2010). We elaborate on this evolutionary theme as we delve into the even earlier history of these two nodulating and nitrogen-fixing bacterial families, with the goal of establishing an evolutionary description of this still incompletely understood symbiosis.

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89.2 THE LEGUMES

The legumes (Fabaceae or Leguminosae) are the third largest family of flowering plants (>19,000 species); only Asteraceae and Orchidaceae are larger (Lewis et al., 2005). The family is monophyletic, but comprises three subfamilies (36 tribes), namely Caesalpinioideae, Mimosoideae, and Papillionoideae, which represent 22%, 10%, and 67%, respectively, of the family (Sprent, 2001). Of the three subfamilies, caesalpinioid legumes are less likely to be nodulated, but this is not because they evolved first (Sprent, 2007). Several caesalpinioid legumes considered to be "basal" are not found in the fossil flora in the early history of the family (Herendeen et al., 1992).

The structure of the flowers of the subfamily members differs significantly (Fig. 89.1), but legumes are incredibly diverse in many other features, including phytochemical profiles, nodulation status, and habitats. Legumes occupy deserts and grass-poor, succulent-rich, dry environments (S-biomes); tropical rain forests (R-biomes); grass-rich prairies and savannahs (G-biomes); temperate ecosystems (T-biomes); and agricultural systems derived from anthropogenic input. Numerous books and papers have described the biology, nodulation, and evolution of the legumes, so we describe only a few salient features in this chapter (Sprent, 2001; Doyle and Luckow, 2003; Lewis et al., 2005).

The legumes are believed to have evolved ca. 65–50 million years ago (Mya) in the late Cretaceous/early Tertiary after the K/T extinction (Lavin et al., 2005). This hypothesis is in part based on the discovery of legume fossils dating from the Paleocene (ca. 66–56 Mya; Herendeen et al., 1992) and also on molecular analysis developed from *matK* and *rbcL* phylogenies (Lavin et al., 2005). The evolutionary migration of legumes southward is postulated to have started north of the Tethys Sea, under warmer climate conditions, after the continents drifted apart (see Doyle and Luckow, 2003; Sprent, 2007). This boreotropical origin, deduced in part from the current habitats of legumes, is consistent with the migration of legumes from an S-biome to the R-, G-, and T-biomes (Lewis et al., 2005). Both molecular and fossil data suggest that legumes rapidly diversified into most of their major lineages within a relatively short period of time (ca. 55–50 Mya) (Herendeen et al., 1992; Doyle and Luckow, 2003; Lewis et al., 2005; Sprent, 2007, 2008). This is approximately the same time that the rest of the angiosperm families were evolving (Lewis et al., 2005). The boreotropical forest migration hypothesis has replaced the older Gondwanan theory in which legumes moved northward from their presumed sites of origin in Africa to South America and then, before the land masses drifted apart 165–145 Mya, to North America. Nonetheless, the boreotropical hypothesis leaves much unexplained, especially with regard to the evolution of legume nodulation (Sprent, 2008; see Chapter 3).

Although nodules have not been found in the fossil record, it is extremely likely that many of the legumes were already developing root nodules inhabited by nitrogen-fixing rhizobia by the time that the major lineages were established. The partnership between rhizobia and legumes is based on a pre-existing and ancient relationship, dating from the early Devonian (416–350 Mya) between arbuscular mycorrhizal fungi (AMF) and plants. The evolutionary origin of the genes important for root nodulation that was derived from the arbuscular-mycorrhizal (AM) symbiosis has been extensively studied and reviewed (Hirsch et al., 2001; Szczglowsky and Amyot, 2003; Streng et al., 2011; and others; see also Chapters 42, 54, 55, 108, 110).

89.3 THE RHIZOBIA

"Rhizobia" is the collective term given to bacteria capable of inducing the development of and then populating nitrogen-fixing nodules (Sprent, 2001). A long-standing debate exists over whether legumes and rhizobia coevolved or whether the plants selected for symbiotic bacteria, but the presence of horizontal gene transfer and the lack of parallel speciation between legumes and rhizobia might argue against a coevolutionary trajectory (Martínez-Romero, 2009; Young and Johnston, 1989).

Up to 2001, only the Alphaproteobacteria in the family Rhizobiaceae had been classified as rhizobia. These bacteria

Figure 89.1 Examples of flowers from the three legume subfamilies. (a) Caesalpinoideae: *Chamaecrista fasciculata* (N.A. Fujishige). (b) Mimosoideae: *Caliandra hematocephala* (A.M. Hirsch). (c) Papillionoideae: *Swainsona formosa* (W. Deng).

were first isolated from root nodules by Beijerinck (1890), and the name *Rhizobium* was suggested shortly afterwards by Frank (1889) (see additional references in Gyaneshwar et al., 2011; see Chapter 88). Until the 1980s, all the legume root-nodulating bacteria (RNB) were classified in the genus *Rhizobium*, which had been divided into fast and slow growers. After it became apparent that significantly greater differences than just growth rate existed, *Bradyrhizobium* (*Bradyrhizobiaceae*) was proposed by Jordan (1982) to encompass the slow-growing species whereas the name *Rhizobium* was retained for the fast growers. Using a molecular clock approach, the split between *Rhizobium* and *Bradyrhizobium* is estimated to have taken place 800–300 Mya depending on the gene analyzed (Turner and Young, 2000).

Rhizobium has also been subdivided into a number of distinct genera. Chen et al. (1988) isolated fast- and slow-growing rhizobia from nodules of soybean (*Glycine soja*) and from soil, and after analyzing cellular composition and by using methods such as DNA–DNA hybridization, serology, and phage typing, proposed a new genus, *Sinorhizobium*. Thus, the fast-growing isolate *Rhizobium fredii* was renamed *Sinorhizobium fredii*. In 2008, however, taxonomists questioned whether *Sinorhizobium* was distinct from the genus *Ensifer*, and the Judicial Commission of the International Committee on Systematics of Prokaryotes (2008) decided that it was not, and thus the name *Ensifer*, which had priority, was retained (see also Chapter 3). Later, other genera were described, including *Azorhizobium* (Dreyfus et al., 1988), which was placed in the family *Xanthobacteraceae*, and *Mesorhizobium* (Jarvis et al., 1997) in the *Phyllobacteriaceae*. The genus *Phyllobacterium* contains three rhizobial species that are closely related to *Mesorhizobium* (Valverde et al., 2005; Mantelin et al., 2006).

Concurrently, additional RNB from Rhizobiales were added to the list: *Devosia neptunia* (family *Hyphomicrobiaceae* (Rivas *et al.*, 2003)); *Ochrobactrum* (Trujillo et al., 2005; Zurdo-Piñeiro et al., 2007) (family *Brucellaceae*); *Microvirga* (Ardley et al., 2012; see Chapter 23); and *Methylobacterium* (family *Methylbacteriaceae*) (Jourand *et al.*, 2004). Each family that contains RNB also houses nonrhizobial species, some of which are pathogenic on animals or plants. For example, most of the *Xanthobacteraceae* are nonnodulating species and many *Brucellaceae* are human pathogens. As another example, *Bradyrhizobium* is closely related to the genus *Apifia*, which includes human pathogens (La Scola *et al.*, 2002).

Other novel species of rhizobia recently isolated include *Shinella kummerowiae* from the herbal legume *Kummerowia stipulacea* in China (Lin et al., 2008). This genus is closely related to *Rhizobium* and the bacteria are often found as nodule occupants and in soil samples, but had not been shown previously to induce nodules. The diversity of organisms that function as RNB is summarized in Figure 89.2.

89.4 THE BETARHIZOBIA

In 2001, two genera of the family *Burkholderiaceae*, *Burkholderia* and *Cupriavidus*, were reported to nodulate legumes, but at that time were not shown to induce effective, that is, nitrogen-fixing nodules on their hosts (Moulin et al., 2001; see Chapter 17). Much of the original work on the *Burkholderia* symbiotic species focused on *B. tuberum* and *B. phymatum*, but it was quickly shown that neither species re-infected the hosts from which they were originally isolated (*Aspalathus carnosa* and *Machaerium lunatum*, respectively). Later studies showed that both genera established nitrogen-fixing nodules with a number of legume hosts. The majority of *Burkholderia* species have been isolated from nodules of Mimosoideae, especially the genus *Mimosa*. *Mimosa* spp. are native to the Neotropics although a few endemics live in the Paleotropics, mainly in Madagascar, and also extend into southeast tropical Africa and India (Lewis et al., 2005; see Chapter 17).

Some *Burkholderia* species are free-living and function as soil-dwelling microbes in bioremediation or act as plant growth-promoting bacteria (PGPB) living as epiphytes on plants (de Bruijn, 2013). Still others appear to be endophytes based on their isolation from both legume and nonlegume hosts following surface sterilization of plant tissues (Compant et al., 2008). However, many of these species have not been rigorously confirmed as either endophytes or PGPB based on experimental studies, with the exception of *B. phytofirmans* (Sessitsch et al., 2005).

Yabuuchi et al. (1992) split *Burkholderia* from *Pseudomonas* homology group II and named the genus for W.H. Burkholder, who isolated the etiological agent of onion rot (Burkholder, 1942). *Burkholderia* species, other than the RNB (shown in Fig. 89.3), are ubiquitous in the environment and are found in soil, rhizosphere, clinical samples, plants, fungus, insects, and animals (Compant et al., 2008). *Burkholderia* species have long been associated with plant and animal pathogenesis, and all of the initial species moved from *Pseudomonas* to *Burkholderia* demonstrated virulence toward numerous plants and animals (Compant et al., 2008). However, efforts have been initiated to differentiate the symbiotic and environmental *Burkholderia* species from the opportunistic, mammalian, and plant pathogens, and have led to the proposal that the former be assigned to a new group/genus because it is phylogenetically distinct from the *Burkholderia* group (Angus et al., 2014, Estrada-de Los Santos et al., 2013, Suárez-Moreno et al., 2012; see also Chapter 17).

89.5 THE ROOT-NODULATING *Burkholderia* SPECIES

To study the evolution of symbiotic genes, Bontemps et al. (2010) prepared a phylogenetic analysis using partial

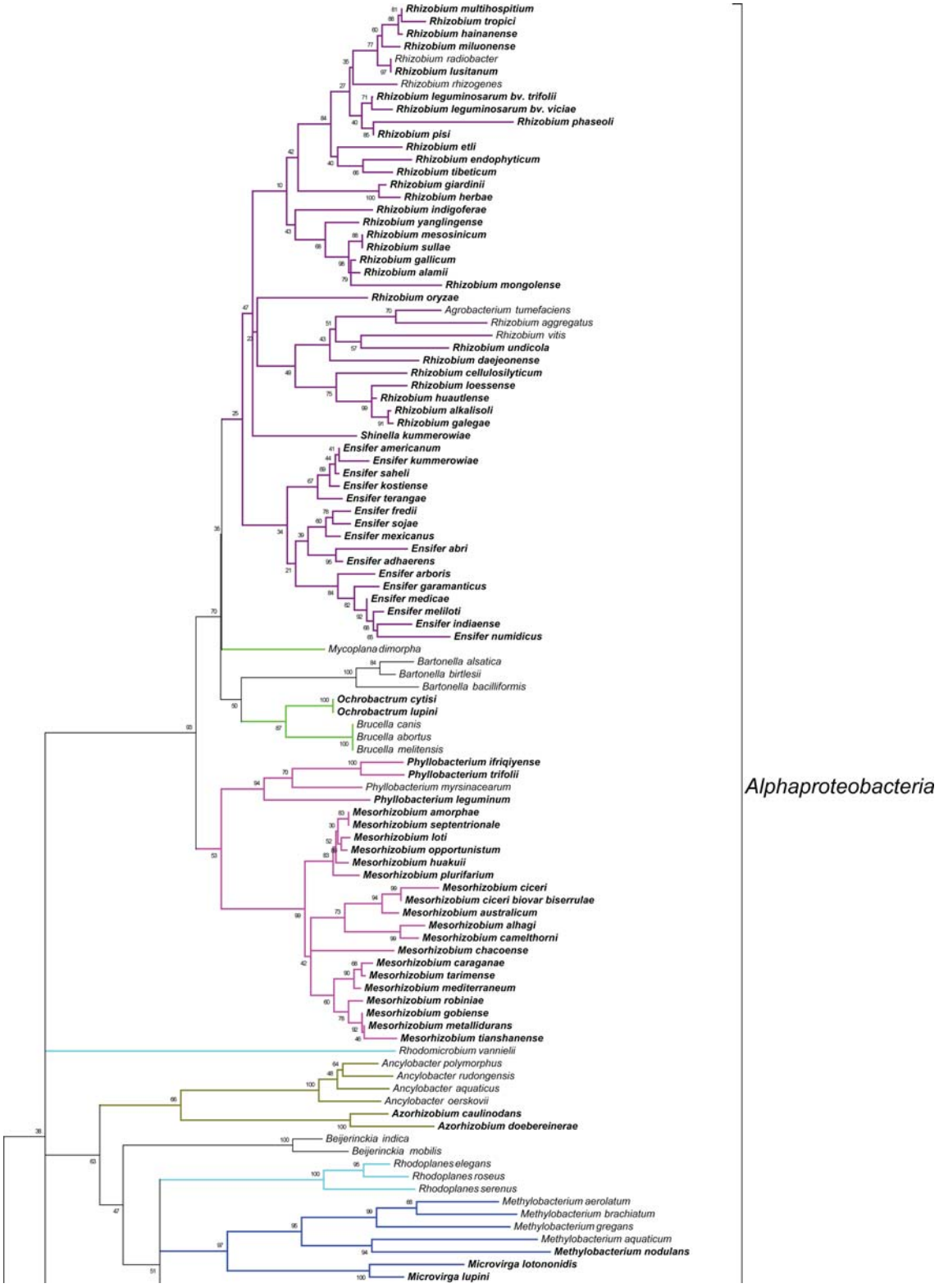

Figure 89.2 Unrooted phylogenetic reconstruction of 16S ribosomal RNA of rhizobial and related nonrhizobial species by Neighbor Joining. Bootstrap percentage after 1000 replications is shown on nodes. Scale bar represents the number of substitutions per site. Colored nodes represent each of the main families with genera containing rhizobia (in bold). Purple, *Rhizobiaceae*; green, *Brucellaceae*; pink, *Phyllobacteriaceae*; olive, *Xanthobacteraceae*; turquoise, *Hyphomicrobiaceae*; blue, *Methylobacteriaceae*; orange, *Bradyrhizobiaceae*; red, *Burkholderiaceae* (see also Chapter 17).

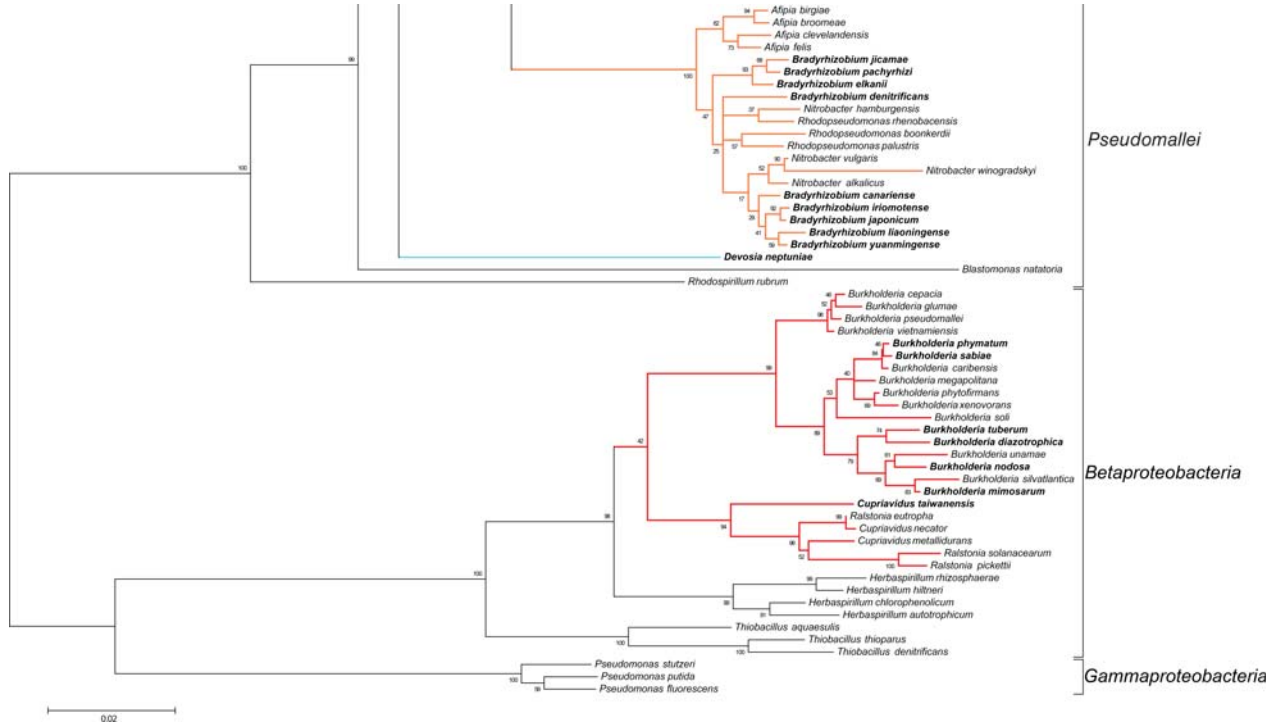

Figure 89.2 (*Continued*)

sequences of the 16S, *recA*, *nodC*, and *nifH* regions of isolates from nodules of 47 species of *Mimosa* spp. growing in various regions of Brazil. All sequences were highly homologous to *Burkholderia* species from seven distinct clusters (clades). Four of the seven clusters observed by Bontemps et al. (2010) contained named species of *Burkholderia* RNB (cluster 2, *B. phymatum*; cluster 4, *B. mimosarum*; cluster 5, *B. nodosa*; and cluster 6, *B. tuberum*). However, three clusters represented novel species (clusters 1, 3, and 7). Based on recent evidence, cluster 3 is closely related to the recently described *B. diazotrophica* (Sheu et al., 2013); one isolate within this cluster, *Burkholderia* sp. mpa3.10 shares over 99% 16S and *recA* sequence homology to *B. diazotrophica* (Walker & Watkin, *unpublished*). Isolates from cluster 7 may be related to the recently sequenced *B. phenoliruptrix* (de Oliveira et al., 2012), and are also closely related to *B. fungorum*. Cluster 1 may contain the recently described *B. symbiotica*, which is the first *Burkholderia* sp. RNB characterized that is not closely related to other Brazilian species (Sheu et al., 2012).

The study by Bontemps et al. (2010) gave us important insight into the evolution of nodulation in *Burkholderia* by showing that it had a long and stable genetic history (Angus and Hirsch, 2010), which is most likely as old as that of the nodulating Rhizobiales. Phylograms reconstructed from partial *nodC* sequences place South American *Burkholderia* symbiotic genes in a monophyletic group that is highly divergent from all other *nodC* sequences and further suggest that any *nod* gene transfer event that occurred must have taken place at least 50–60 Mya (Bontemps et al., 2010). This transfer of symbiotic genes coincides with the period when the legumes themselves were diversifying into their major lineages (Fig. 89.4).

The legume genus *Mimosa* is almost exclusively nodulated by species of *Burkholderia* (see also Chapter 17). *Mimosa* most likely followed a boreotropical migration from north to south and this theory is supported by fossil evidence in North America and Europe, but palynological data have also been collected in Africa (Herendeen et al., 1992). However, disjunct populations of *Mimosa* in South Eastern Africa, Madagascar, and India may have originated from long-distance oceanic dispersal (Simon et al., 2011). Some of these introduced populations might be coincident with the southern hemisphere locations of the isolated RNB *Burkholderia* species (Fig. 89.3).

In contrast to the South American *Burkholderia* species, the South African *B. tuberum* strains nodulate papillionoid legumes. This nodulation preference is correlated with nodulation (*nod*) and nitrogen-fixation genes (*nif*) gene sequence organization, which differ between the South

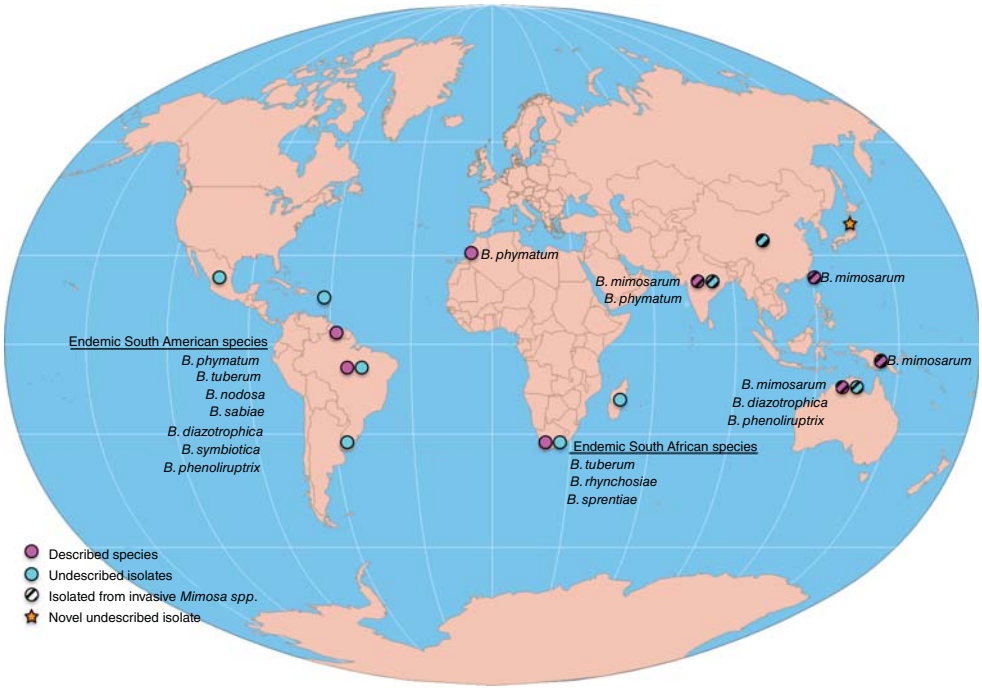

Figure 89.3 Distribution of characterized type strains and undescribed isolates of *Burkholderia*. Note the mostly southern hemisphere distribution of the strain isolations. Strain CCGE1002 (Mexico) is described as *B. tuberum* in Mishra et al., (2012).

African and South American RNB. One hypothesis posits that the establishment of *Mimosa* from long-distance oceanic dispersal may have led to the introduction of *B. tuberum* into South Africa, where it could have lost the ability to nodulate *Mimosa* in favor of the endemic Papillionoideae population following the lateral transfer of local *nod* genes (from alpha rhizobia?). *Mimosa* introduction has been observed in Australia with the accidental importation of South American *Burkholderia* species, including *B. diazotrophica* and *B. mimosarum* (Walker and Watkin, *unpublished*), on introduced *M. pigra* seeds (Parker et al., 2007).

The idea of introduced *Mimosa* species, however, does not adequately explain the distinct lineages of *Burkholderia* species found in South Africa that nodulate papillionoid legumes. The first lineage described is typified by the type strain *B. tuberum* STM678^T, originally isolated from *A. carnosa*, whereas a second lineage contains the recently described *B. rhynchosiae* (De Meyer et al., 2013a), which nodulates species of *Rhynchosia* (Garau et al., 2009). So far, no information is available about *nod* and *nif* gene organization in *B. rhynchosiae*, nor about how these symbiotic genes compare to those of *B. tuberum* STM678^T except for the fact that the *nodA* genes are 96% identical (Garau et al., 2009). Another lineage, *B. sprentiae*, is more closely related to *B. tuberum* STM678^T, but it nodulates *Lebeckia* spp. (De Meyer et al., 2013b). Numerous South African species have been recently isolated and many are in the process of being described (Beukes et al., 2013; De Meyer et al., 2014). Such studies will increase our understanding of this group of *Burkholderia* spp.

89.6 REGARDING THE EVOLUTION OF NODULATING *Burkholderiaceae* AND *Rhizobiaceae*

A limited fossil record exists for bacteria, except for cyanobacteria where fossils resembling these organisms have been reported from the Archean period of Earth history (Schopf, 2006; Javaux et al., 2010). Nonetheless, questions remain as to their actual identity and whether or not cyanobacterial photosynthesis evolved that early. In any case, most agree that marine bacteria probably evolved about 3.5 billion years ago (Gya). Undisputed microfossils from 1.2 Gya have been found (Horodyski and Knauth, 1994) (Fig. 89.4). Due to the debate about the exact nature of the fossils from 3.5 Gya, conclusions regarding bacterial evolution are based mainly on geochemical evidence, the detection of biomarkers, and isotope ratios (Fischer, 2008). The accumulation of such information has strongly suggested that bacteria achieved a terrestrial lifestyle (became terrabacteria) some 2.7–2.6 Gya (Watanabe et al., 2000) (Fig. 89.4). Moreover, it appears that many prokaryotic lineages were already evolved as early as 2.7–2.5 Gya, supporting the even older history for the earliest bacteria.

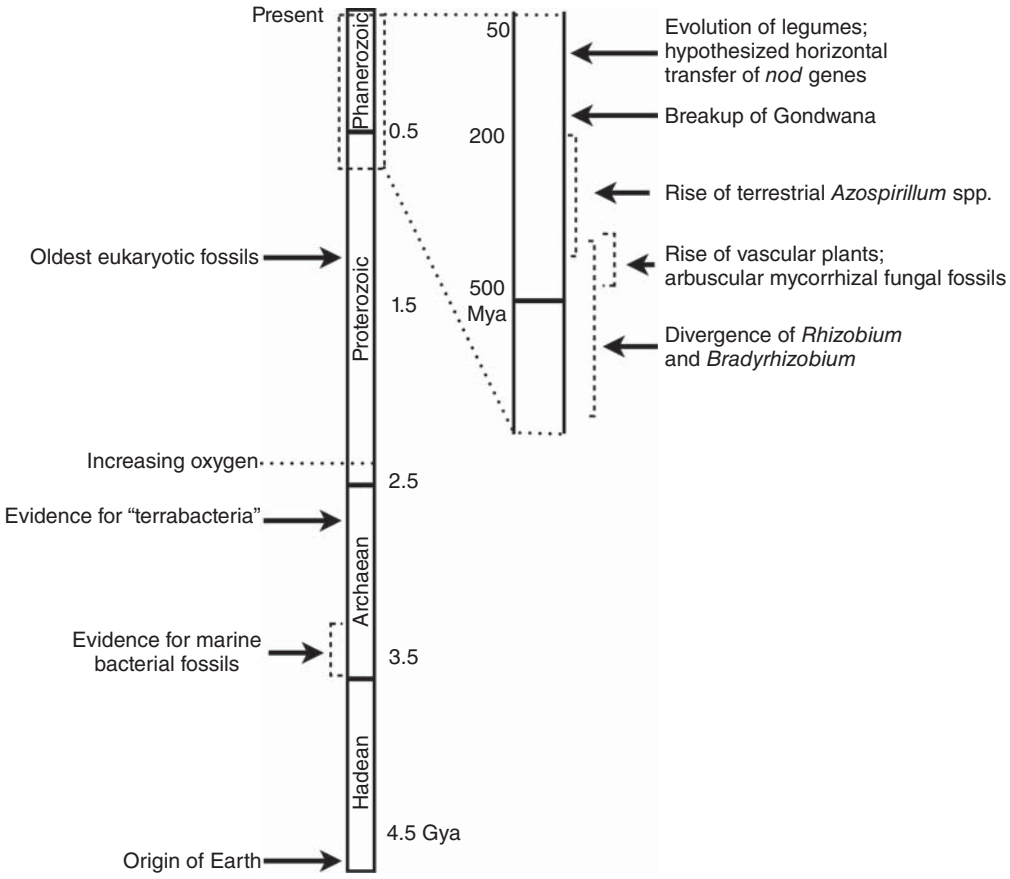

Figure 89.4 Timeline showing some key points in the evolution of the legumes and their associated nitrogen-fixing bacteria. (*Source:* Modified from Fischer (2008).)

More recently, genome analysis has been used to study bacterial evolution. Wisniewsky-Dyé et al. (2011) proposed the divergence of terrestrial azospirilla from their aquatic *Rhodosporillaceae* relatives had occurred ca. 200–400 Mya based on their distinct genomes. Almost half of the terrestrial azospirilla genomes appear to have been derived by horizontal gene transfer from Rhizobiales and other Alphaproteobacteria (greatest percentage) and also from Burkholderiales, suggesting that these groups evolved long before *Azospirillum* diverged. Therefore, Rhizobiales and Burkholderiales were likely to have been among the bacterial lineages that colonized the land prior to the evolution of the vascular plants, the vast majority of which established a symbiotic association with AMF (Fig. 89.4). Both fossil and molecular evidence date the AMF association with plants to have been present ca. 430–350 Mya in the early Devonian (Simon et al., 1993; Taylor et al., 1995). Because many of the plant genes involved in establishing the AM symbiosis and the nitrogen-fixing nodule are conserved and also because the initial microbial signal molecules are chemically related (see references in Hirsch and Fujishige, 2012; see Chapters 55, 110), the idea that nitrogen-fixing rhizobia coopted the ancient mycorrhizal signaling pathway to ensure root cell entry and subsequent nodulation for the proliferation and protection of the bacteria is well accepted.

Thus, this brings up the question as to whether ancestral Rhizobiales and/or Burkholderiales could have been coparticipants in the origin of both plant–mycorrhizal associations and nitrogen-fixing symbioses. For the Betaproteobacteria, the answer is probably yes based on *Burkholderia* symbionts being present within AMF hyphae. *Candidatus Glomeribacteria gigasporarum* (CGG) is an obligate endobacterium that lives surrounded by a membrane within the cytoplasm of AMF cells, which themselves are obligate biotrophs and dependent on their plant hosts (Jargeat et al., 2004). However, no evidence for *nif* genes was uncovered (Jargeat et al., 2004), suggesting that the CGG ancestor was unlikely to be a diazotroph although it is sister to free-living *Burkholderia* species that fix nitrogen as well as to *B. rhizoxinica* HKI 0454^T (Ghignone et al., 2004), an endophyte of the phytopathogenic fungus *Rhizopus*, the causal agent of rice-seedling blight (Partida-Martinez et al., 2007). In spite of its small genomes and lack of *nif* genes, some 250,000 CGG cells are estimated to live within one

fungal cell (Bianciotto et al., 1996) and at least 20,000 per spore (Lumini et al., 2007), strongly suggesting that these microbes provide benefits to their host. Indeed, the bacteria were found to improve the fitness of Gigasporaceae by promoting presymbiotic hyphal expansion and branching (Lumini et al., 2007).

Based on studies of various betaproteobacterial genomes, the difference in mutation accumulation in CGG differs from free-living *Burkholderia* species and from the *Burkholderia* endosymbionts of *Rhizopus*, strongly suggesting an ancient interaction with the fungus (Castillo and Pawlowska, 2010). Similar to insect symbionts, CGG and *B. rhizoxinic*a have reduced genomes, but the fungal pathogen has a large number of genes associated with virulence (Lackner et al., 2011). Moreover, *B. rhizoxincia* can be grown in culture and re-infect its fungal host (Moebius et al., 2014). In contrast, CGG is unable to synthesize several essential amino acids and vitamins, and is totally dependent on its host for carbon, nitrogen, and phosphorous (Ghignone et al., 2012). The complex network seen in this tripartite symbiosis strongly suggests a different pattern of molecular evolution than that observed in the free-living *Burkholderia* species or *B. rhizoxincia* (Castillo and Pawlowska, 2010). Whether or not an ancient *Burkholderia*–AMF–plant symbiosis is the ancestor of the nodulation pathway (see Chapters 51, 59, 110) may be difficult to prove, but it is not clear whether any better candidate presents itself. To our knowledge, the evidence for a Rhizobiales endosymbiont living in association with AMF cells has not been reported.

89.7 FINAL COMMENTS AND PERSPECTIVES

Strong evidence for distinct *Burkholderia* populations in South America and Southern Africa exists, but so far not much is known about the Indian *Burkholderia* populations (Fig. 89.3). The Indian *Burkholderia* RNB may be closely related to RNB isolated from *Dalbergia* legume nodules in Madagascar although this strain was identified at the time as *B. cepacia* and lacks *nod* genes (Rasolomampianina et al., 2005). Although *Burkholderia* strains have been isolated from nodules of legumes growing in Australia, so far no *nod* genes have been detected in these (R. Walker, *unpublished*). For China and Europe, no reports of *Burkholderia* RNB from **endemic** legumes have been described. Clearly more isolations need to be made. Thus, it appears that Southern Africa and South America are the major centers for *Burkholderia* evolution (see also Chapter 17).

To explain these apparently disparate centers of origin of the RNB *Burkholderiacae*, it is important to remember that ca. 200 Mya, South America, Africa, India, and Australia were all part of the single continent Gondwana. If *Burkholdericeae* and *Rhizobiaceae* date from this time, and based on timing of the divergence of *Bradyrhizobium* and *Rhizobium* and the presence of *Burkholderia* in AMF (Fig. 89.4), it is a formal possibility that the two families were members of the Gondwanan microflora. Thus, it can be deduced that certain *Burkholderia* species such as *B. tuberum* originated when South Africa and South America were still connected, which might explain the disparate localization of *B. tuberum* with the mimosoid (South American) and papillionoid (South African) legumes after the continents split. The example of a South American *B. tuberum* is the strain *Burkholderia* CCGE1002, which was isolated from nodules of *Mimosa occidentalis* growing in Mexico and has characteristics typifying both the South African and South American strains (Mishra et al., 2012; Ormeño-Orrillo et al., 2012). Strain CCGE1002 is *B. tuberum* based on a concatenated sequence of 16S RNA and four housekeeping genes (Agapakis et al., *unpublished*), but not if based on *nodA* or *nifH* (Mishra et al., 2012), However, in contrast to South African *B. tuberum* STM678^T and *B. tuberum* related strains such as *B. rhynchosiae*, *B. dilworthii*, and *B. sprentiae* (De Meyer et al., 2013a,b, 2014), CCGE1002 has a *nod-nif* gene arrangement upon a symbiotic plasmid that is similar to that of other *Mimosa*-nodulating *Burkholderia* strains except it has an integrase gene terminating the operon (Agapakis et al., *unpublished*). In addition, the *nod* and *nif* genes of the Mimosoideae-nodulating *Burkholderia* strains are not closely related to those of the Papillionoideae-nodulating South African *B. tuberum* strains (Mishra et al., 2012). Bontemps et al. (2010) placed the acquisition of the symbiotic genes by RNB at about the same time as when the legumes were diversifying. We propose that the presence of different legume subfamilies, Mimosoid versus Papillionoid, as well as the influence of different biogeographies on the two once connected continents facilitated the divergence of *Burkholderia* RNB into separate lineages, accounting for the *Mimosa*-nodulating strain CCGE1002 versus the Papillionoideae-nodulating *B. tuberum* STM678^T and related species (see also Chapter 19).

Finally, which of the RNB evolved first, the alpharhizobia or betarhizobia? If *Burkholderia* species inhabited fungal cells at the time of the emergence of the vascular plants, this strongly suggests their ancient origin. The timeline implies that *Burkholderia* may have associated with plants some 500 Mya via the AM symbiosis, and in the process, recruited the capacity for an intimate interaction with the plant from AMF via an unknown mechanism. This hypothesis suggests that *Burkholderia* could have been the source of the nodulation genes via lateral transfer of genes if a nonreduced genome ancestor associated with the fungi. However, an alternative hypothesis, as previously mentioned (Hirsch et al., 2001), is that the original source of these genes was outside either *Burkholderiaceae* or *Rhizobiaceae* because of their lower G + C content compared to the rest of the genome. For both groups of RNB, the genome G + C

content is higher overall than that of the nodulation genes. Potential recruitment of *nodB* and *nodC* from other bacteria or fungi is a distinct possibility, but more elusive is the source of the gene-encoding NodA, the protein responsible for adding a fatty acyl chain onto the resulting free amino group of the nascent Nod factor.

Based on the isolations of *Burkholderia* RNB so far (Fig. 89.3), it appears that the RNB group is confined to the southern hemisphere except for CCGE1002, which was isolated from nodules in Mexico, as well as some undescribed isolates from China. However, based on fossil data, the distribution of many legume genera is very different from what it was predicted to be 65 Mya. The greatest diversity at the generic level then and now is in tropical America and Africa/Madagascar (Herendeen et al., 1992). Coincidentally, this distribution overlaps with that of the *Burkholderia* RNB. Of the North American fossil flora, many extant relatives are now restricted to South America possibly as a result of climatic constraints (Herendeen et al., 1992). A more extensive focus on *Burkholderia* RNB and their hosts is needed to obtain a more complete knowledge of legume and symbiont biogeography.

Exploring the origins of nodulation provides insight into the evolution and diversity of symbiosis more broadly. Interactions between plants and microbes have an ancient and important history. The role of the *Burkholderia* RNBs in that history, especially in the southern hemisphere, demonstrates the potential value of these strains in future agricultural applications as well as in our broad understanding of ecological and evolutionary relationships (see also Chapter 17).

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Chapter 90

Agronomic Applications of *Azospirillum* and Other PGPR

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90.1 INTRODUCTION AND DISCUSSION

90.1.1 Plant Growth Promoting Rhizobacteria (PGPR)

The rhizosphere is the area of soil influenced by plant roots. It is composed of microbial populations that are somehow different than the rest of the soil populations, generally denominated as the "rhizosphere effect" (de Bruijn, 2013).

Among the microorganisms inhabiting the rhizosphere it is possible to isolate a wide variety of viruses, bacteria, protozoa, and fungi. Some microbial species are capable of promoting root and plant growth (de Bruijn, 2013). Other species are neutral and some are deleterious to plant growth (Helman et al., 2011). In this chapter, we deal with some well-characterized bacteria that are known to promote root and plant growth directly and have good potential to be commercially applied for increasing the yields of agricultural crops; they are generally denominated as PGPR (Plant Growth Promoting Rhizobacteria; de Bruijn, 2013). PGPR with potential to directly promote crop yield include species from the *Azospirillum, Herbaspirillum, Gluconacetobacter, Burkholderia, Pseudomonas,* and *Paenibacillus* genera, among others. The genus *Azospirillum* is the most widely commercially used in agriculture, especially in South America, and will be reviewed in more detail. We are not including in this chapter indirect biological control for plant growth promotion (see de Bruijn, 2013).

The genus *Herbaspirillum* (beta proteobacteria) comprises several diazotrophic species (see Chapter 93). Some colonize root surfaces but are also capable of endophytic and systemic colonization of several plant species (Reis et al., 2007). An additional nitrogen-fixing endophyte PGPR, which is capable of colonizing sugarcane and other plants, is *Gluconacetobacter diazotrophicus* (alpha proteobacteria) (Reis et al., 2007). Nitrogen-fixing, plant-associated *Burkholderia* (beta proteobacteria) represent great potential for agrobiotechnological applications (Caballero-Mellado et al., 2007; see Chapters 17 and 89). The bacterial genus *Pseudomonas* (gamma proteobacteria) also comprises many bacterial species with agricultural importance. Some such as *P. syingae* are plant pathogenic bacteria of important crops and have been extensively studied. Other *Pseudomonas* species are involved in disease suppression and/or direct plant growth promotion (Glick et al., 2007; de Bruijn, 2013). The genus *Paenibacillus* comprises over 30 species of facultative anaerobes and endospore-forming, periflagellated, heterotrophic,

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low G + C gram-positive bacilli (see Chapter 92). *Paenibacillus polymyxa* is of biotechnological potential, mostly due to its biological control properties (Lal and Tabacchioni, 2009). The *Azospirillum* genus (Baldani et al., 2005) belongs to the alpha proteobacteria and comprises free-living, nitrogen-fixing, vibrio- or spirillum-shaped rods that exert beneficial effects on plant growth and the yield of many agronomically important crops. Azospirilla are able to fix nitrogen in association with plants but most inoculation experiments and systems evaluated so far indicate that nitrogen fixation does not play a major role in plant growth promotion (Helman et al., 2011).

The PGPR species mentioned above have been subjected to extensive studies to elucidate their colonization capabilities of surfaces and of interior of plants, and their physiological and molecular mechanisms involved in promoting plant growth (see de Bruijn, 2013). Genomes of several species are now available and the extensive knowledge acquired will possibly enable a more comprehensible biotechnological use of the bacteria as commercial products in the near future.

The main issues that have been investigated on mechanisms of direct plant growth promotion and are commonly reviewed in the literature remain to some extent controversial and there are only few clear demonstrations of their involvement, especially under more natural field conditions (Helman et al., 2011, Cassán and García de Salamone, 2008a). In this chapter, the PGPR effect will be evaluated with emphasis on the applied aspects of the *Azospirillum*–plant interactions.

One of the most pronounced effects of inoculation with azospirilla and other diazotrophic PGPRs on root morphology is the proliferation of root hairs as observed in several grasses, cereals, and legumes. However, morphologically, the proliferation and morphology of root hairs is not similar when comparing inoculation with different PGPR species (Dobbelaere and Okon, 2007). Inoculation can also promote the elongation of primary roots and increase the number and length of lateral roots. Again, when observed in detail, the morphological effects differ with the different PGPR inoculum. The most clear and distinct effects on root morphology have been observed after inoculation with Azospirillum species and strains (Dobbelaere and Okon, 2007).

The morphological effects on roots are generally dependent on the inoculum concentration and are consistent with the exogenous indole-acetic acid (IAA) levels secreted by *Azospirillum,* indicating that they are mainly due to the production and secretion of IAA by the bacterium (see also Chapter 91). There is evidence that secretion of cytokinins and gibberellins by the bacteria is also involved, but most important could be the IAA/cytokinin ratio (Spaepen et al., 2009). Another diffusible molecule involved, as in the case of *A. Brasilense,* is nitric oxide (NO), a key signaling molecule involved in a wide range of functions in plants (Molina-Favero et al., 2008; see also Chapter 64).

The described effects on root growth and activity mainly in the case of *Azospirillum* result in enhanced mineral and water uptake from the soil by the inoculated roots. This has been repeatedly demonstrated under greenhouse and field conditions in various important crops such as maize and wheat (Dobbelaere and Okon, 2007).

Biological nitrogen fixation (BNF) in diazotrophic PGPR inoculated plants (maize, wheat and other grasses) as well as in sugarcane has been investigated in detail by various techniques including the acetylene reduction assay (ARA), the ^{15}N dilution technique, $^{15}N_2$ fixation, ^{15}N natural abundance, and Kjeldhal N-content measurements (Dobbelaere and Okon, 2007). In most grain and forage crops the contribution of nitrogen fixation by *Azospirillum* has been estimated to be no more than 10 kg N/ha/year. The quantities of fixed nitrogen supplied to some cultivars of sugarcane and rice by *G. diazotrophicus* and other PGPR have been estimated to be as high as 50% of the required plant nitrogen (Reis et al., 2007). Since there are many other diazotrophs colonizing the surface and internal tissues of sugarcane roots and rice, it has been difficult to assess the specific contribution of diazotrophic bacteria in the inoculum (Fibach-Paldi et al., 2012).

Some of the unresolved questions for agronomic interactions of PGPR and plants deal with PGPR colonization dynamics, whether PGPR are continuously colonizing and promoting growth of developing roots or alternatively, the major effects are at early stages of root development. Comparable questions are whether nitrogen fixation by the association is continuously taking place or especially at certain stages of plant growth such as inflorescence and grain filling.

Under field conditions, the success of inoculation depends on achieving a high number of bacteria on the root surface and/or the root tissue. There is a need for bacterial colonization in relatively high numbers (10^6–10^7 per g or cm of plant tissue) in order to obtain a significant impact on plant growth promotion derived from BNF, phosphorous solubilization, or disease control (Helman et al., 2011; Spaepen et al., 2009). P-solubilization and BNF are clearly observed and measured in pure cultures when there are $\sim 10^{10}$ cfu/ml; very seldom, high numbers (above 10^4–10^5 cfu) have been counted in plant tissues in the field.

The relevance of PGPR endophytic colonization and its possible advantages in comparison to colonization in the rhizosphere remain to be convincingly demonstrated mainly under field conditions. So far, researchers consider it somehow more logical that endophytic colonization would be more efficient for plant growth, but this needs more investigations (see de Bruijn, 2013). Recent detailed studies support the observations that dual inoculation of *Azospirillum* and some PGPR in legumes (alfalfa, beans, vetch, soybeans and chick peas among others) results in increased production of plant flavonoids and enhanced capacity to induce *Rhizobium nod*-genes expression

(Dardanelli et al., 2008; see Chapter 50). The presence of *Azospirillum* in the legume rhizosphere activates the hydrolysis of conjugated phytohormones and flavonoids in the legume root tissue, thus leading to release of compounds in their more active forms. Results from co-inoculation experiments suggest that IAA and NO produced by *A. brasilense* are key components of enhancement of secretion of *nod*-gene inducing flavonoids in the roots (Dardanelli et al., 2008; Star et al., 2012; Cassán et al., 2009; Hungria et al., 2013).

90.1.2 Field Experiments

Extensive field inoculation experiments with *A. brasilense* carried out in Israel in the 1980s (Okon et al., 1988) clearly show an average significant increase in crop yield of maize, wheat, sorghum, and other forage grasses. In these cases, the seeds were inoculated with freshly prepared peat inoculants (Okon and Labandera-González, 1994; Helman et al., 2011; Cassán and García de Salamone, 2008a).

In earlier field experimentation, when the plant growing properties of *Azospirillum* were considered as derived from BNF and the number of bacteria in the inoculant was not carefully monitored, the commercial exploitation of *Azospirillum* was limited. At this stage, inoculation responses were considered as inconsistent. Further developments emphasize the importance of research and development for improved formulations, the existence of a regulatory frame guaranteeing inoculant quality, and also the need for a network of communication and diffusion systems, with a unique message for the farmers, in order to achieve good field results.

More recently (1998–2013), there have been hundreds of reports of field inoculation experiments utilizing mainly *A. brasilense* liquid or peat-based commercial inoculants, under varied climatic and soil conditions, mainly in Argentina, Uruguay, Brazil, and Mexico (Fuentes-Ramirez and Caballero-Mellado, 2005; Cassán and García de Salamone, 2008b), and also in other parts of the world (Okon and Labandera-González, 1994; Helman et al., 2011).

Statistically significant increases in crop yield have been obtained with intermediate and adequate levels of N, P, K, microelements and water, with high bacterial concentrations (10^9 cfu per g or ml) and inoculants of good physiological status (Spaepen et al., 2009). These inoculant products have been increasingly approved and registered by government agencies, inoculant companies, associations, and official agricultural research institutions (Helman et al., 2011; see also Chapter 101). Thus, based on extensive field experimentation with proper agronomic design and accurate statistical evaluation of crop yield parameters, it is well accepted that the inoculation practice has the potential to promote crop yield in fields. Following the reported success of the PGPR inoculants, there has been an extension of research and inoculant quality controls for these microorganisms and at the same time different companies initiated development and commercialization of products. Commercial inoculants have been regularly applied in recent years; there has been a surge in the inoculation practice with *Azospirillum*, with estimations of about 2–2.5 million doses used in 2012 in Brazil, mainly in maize (Hungria et al., 2010, 2013). The MERCOSUR region was especially receptive to these developments.

90.1.3 Field Experimentation in Uruguay: A Test Case for Development of PGPR Inoculants

In 1990, the Department of Soil Microbiology of Uruguay MGAP, [Department of Soil Microbiology, General Direction of Natural Renewable Resources, Ministry of Livestock, Agriculture and Fisheries (Departamento de Microbiología de Suelos – Dirección General de Recursos Naturales Renovables, Ministerio de Ganadería Agricultura y Pesca, Montevideo, Uruguay – DMS-DGRNR MGAP in the Spanish acronyms)] started a new research line on PGPR emphasizing on *Azospirillum* under controlled and field conditions in legumes and gramineous plants. Preliminary results proved to be very erratic, and because of this MGAP decided to invite Dr. Yaacov Okon in 1992–1993 to analyze available data on PGPR and develop suitable techniques for *Azospirillum* inoculant formulation and quality control. During the consultancy, the first regional workshop on *Azospirillum* took place in August 1993 (Okon and Labandera-González, 1994). The aim of the emerging PGPR project was to enlarge the use of inoculants to other crops following the success of using rhizobial inoculants for legumes.

In 2004, a technical cooperation agreement was signed between the MGAP and Lage & Cia for the validation of a liquid formulation based on *Azospirillum* (Graminosoil brand). One of the relevant aspects in the working procedure was the methodology employed in the trials, where in addition to evaluating crop response to inoculation (dry matter production and grain yield), rhizospheric and endophyte *Azospirillum* determinations were always performed, comparing non-inoculated controls with inoculated treatments, to determine the plant–bacteria relationship and to have better elements for the interpretation of the results.

It is important to note that initial research work on *Azospirillum* in Uruguay focused on studying the bacteria's plant growth promoting and stimulant effects mainly under low levels of nitrogen fertilization. Nevertheless, mainly in the last decade, grain crop production systems in Uruguay were using relatively high levels of chemical fertilizers, with better adjustment to crop requirements. Consequently, it was understood that *Azospirillum* inoculants should aim to increase crop yields rather than intending a substantial decrease in the doses of chemical fertilizers. For this reason, recent field trials were conducted following actual

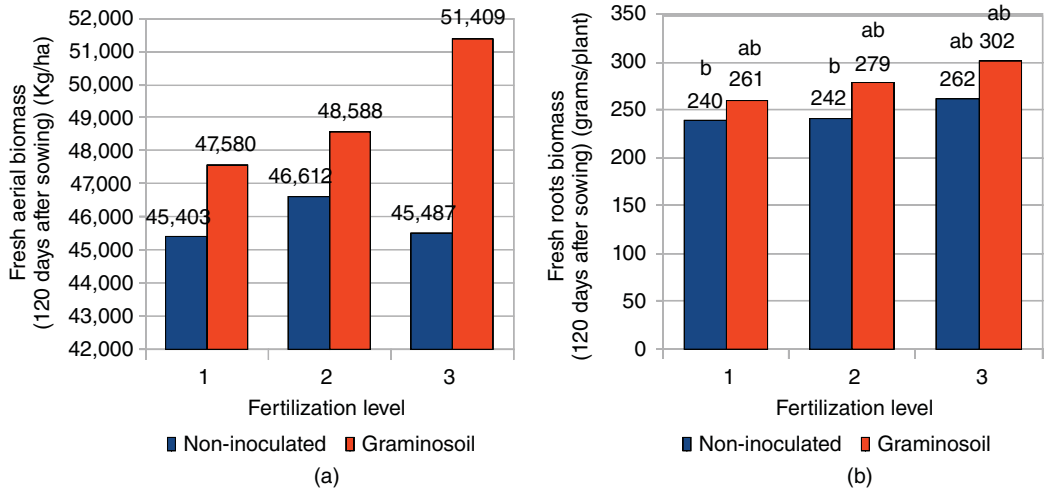

Figure 90.1 Fresh above-ground maize biomass (a) and fresh root according (b) to seed treatment and level of fertilization. Fertilization levels: 1 – no fertilization (neither at planting nor 60 days after), 2 – 0-21/23-0 130 kg/ha at planting; no fertilization 60 days after planting, 3 – 0-21/23-0 130 kg/ha at planting; urea 120 kg/ha 60 days after planting (Casaretto and Labandera, 2008).

fertilization recommendations in terms of doses and ways of application. One of the main production factors that influence yield expression in dry crops in Uruguay is the water regime, since it varies extensively within and between seasons. For this reason, it is often difficult to optimize yield, since although there is a good adjustment of management factors (genetics, length of fallow, planting date, sowing technology and fertilization), rainfall ends up being crucial, either by lack or excess of rain at key moments. Knowing the mechanisms of action of *Azospirillum* and its ability to stimulate root growth, it was considered that *Azospirillum* inoculation could contribute to increase, but most importantly, stabilize yields.

Evaluations of Graminosoil application in maize and sorghum were performed in greenhouse and commercial fields during four consecutive seasons (2003/2004, 2004/2005, 2005/2006 and 2006/2007) (Figs. 90.1–90.4). The evaluated parameters were *Azospirillum* concentration in the inoculant and inoculated seed, *Azospirillum* concentrations in the rhizosphere and inside the root (endophytes), crop establishment, aerial and root dry matter at different physiological stages, and grain yield. Considering the expected responses, these determinations were performed frequently during the crop cycle.

In all experiments the liquid inoculant used had a high azospirilla concentration (10^9 cfu/ml), recovering 10^4 cfu/maize seed, both in non-treated seeds and those treated with chemical fungicides (Carbendazim 250 g/l + Thiram 250 g/l).

Five maize greenhouse trials were conducted, obtaining increases of 30–40% in root biomass and 14–27% in above ground biomass due to inoculation. Similar responses were also obtained in sorghum (C. Labandera, unpublished).

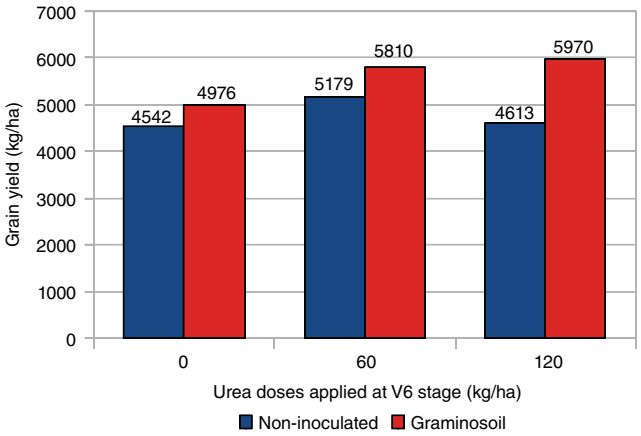

Figure 90.2 Production of maize grain yield in response to inoculation and urea doses at V6. Fertilization at planting: Urea 60 kg/ha + 18-46-0 60 kg/ha. Urea at V6 stage: 1 - none, 2 - 60 kg/ha urea. 3 - 120 kg/ha urea (Hoffman et al., 2008).

In maize field trials there was an increase in the number of bacteria due to inoculation, both in the rhizosphere and inside the roots, of about 2–5 times with respect to the uninoculated control. Following inoculation of maize roots and the aerial part, biomass was significantly increased at different growth stages. Increased production of roots has a direct effect on water and nutrient uptake in inoculated crops. Furthermore, there is an additional value of increased root and stubble input to the system, allowing higher organic matter levels and improving the physical conditions of the productive soil layers, especially in no-tillage systems (C. Labandera, unpublished; Helman et al., 2011).

Figure 90.1a and b shows the results obtained in the field trial performed in 2004/2005 season. In this trial, the greatest differences in aerial biomass production were obtained in

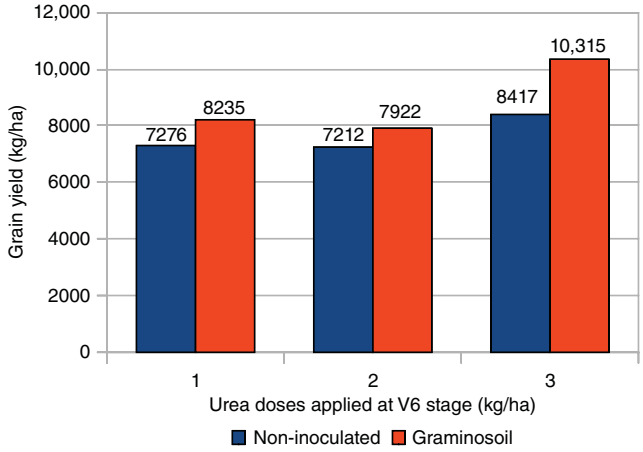

Figure 90.3 Production of maize grain yield in response to inoculation and urea doses at V6. Fertilization at planting: 0-21/23-0 200 kg/ha + 18-46-0 150 kg/ha + Kcl 80 kg/ha. Urea at V6 stage: 1 - none, 2 - 60 kg/ha, 3 - 120 kg/ha (Hoffman et al., 2008).

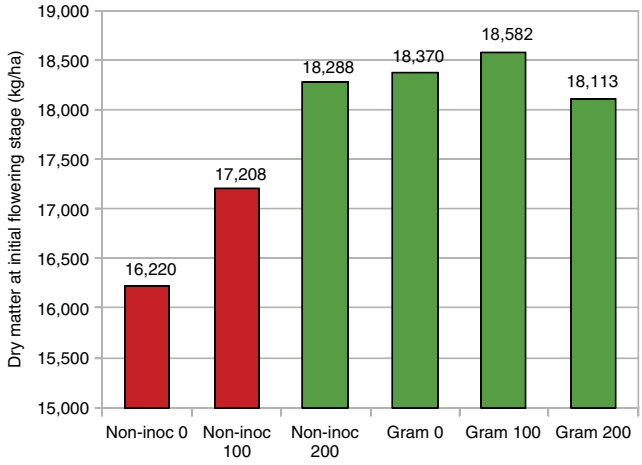

Figure 90.4 Aerial dry matter production of maize at the beginning flowering. Fertilization at planting: urea 50 kg/ha. Urea at V6 stage: 1 - none, 2 - 100 kg/ha, 3 - 200 kg/ha. gram-Inoculated with GRAMINOSOIL, Martino, M. personal communication 2008.

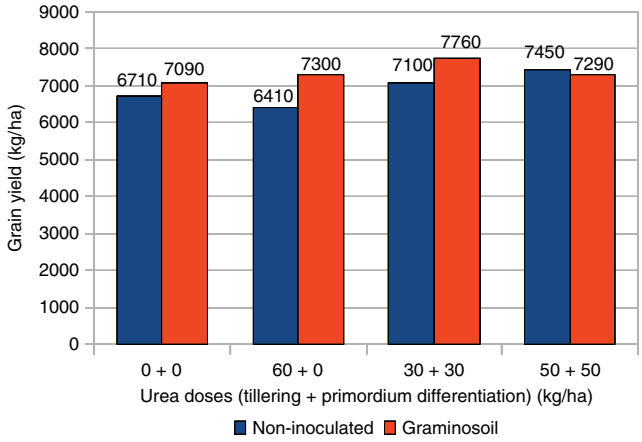

Figure 90.5 Rice yield response to inoculation and doses of urea. Fertilization at planting: 18-46-0 150 kg/ha. Urea at tillering and initial reproductive stage: 1 - none, 2 - 60 kg/ha at tillering, 3 - 30 kg/ha at tillering and 30 kg/ha at initial reproductive stage, 4 - 50 kg/ha at tillering and 50 kg/ha at initial reproductive stage. Chebataroff, N et al personal communication 2007.

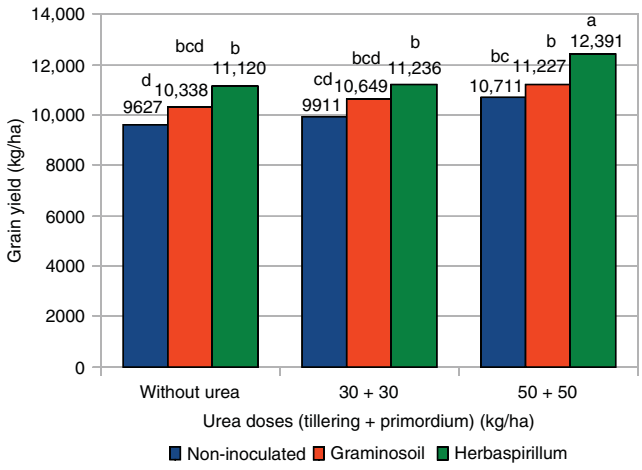

Figure 90.6 Rice yield response to inoculation and doses of urea. Fertilization at planting: 18-46-0 130 kg/ha. Urea at tillering and initial reproductive stage: 1 – none, 2 - 30 kg/ha at tillering and 30 kg/ha at initial reproductive stage, 3 - 50 kg/ha at tillering and 50 kg/ha at initial reproductive stage. Chebataroff, N et al., personal communication, 2009.

the inoculated treatments with higher doses of fertilizer; this would indicate a more efficient use. Moreover, inoculated plants without fertilization reached yields similar to those of the uninoculated control, with higher levels of fertilizer.

Other field trials to evaluate Graminosoil were conducted by private consulting firms in different crops (Figs. 90.2–90.6).

Although interaction in dry matter production and grain yield was not significant, inoculation with *Azospirillum* always maintained higher levels of biomass and grain yield, particularly with high doses of nitrogen (Fig. 90.2).

Increases in maize grain yield at higher levels of N fertilization (Fig. 90.3) and shoot dry matter yield (Fig. 90.4) were obtained in two more field experiments.

Yield increases in rice plots were obtained when seeds were inoculated with Graminosoil and showed to be important in productive and economic terms. Actually, this line of work includes agronomic evaluations of ENDO-RICE inoculant, formulated with a native strain of *Herbaspirillum* (Punschke, and Mayans, 2011).

In conclusion, there has been for several years a clear consistent plant growth promotion and yield increases of important crops in Uruguay, following inoculation with commercial formulations of PGPRs.

90.1.4 Inoculant Production

Following the extensive basic and applied research on PGPR, inoculants for more than 30 years (Helman et al., 2011) commercial inoculant products, field experimentation and international quality control procedures have been established (see also Chapter 101).

The inoculant production process starts with the scaling up of recommended strain(s) for the target crop, according to the institution of reference in each country, when required. The pure cultures are normally maintained at −80 °C, delivered in agar slant tube cultures growing on the appropriate medium. Scaling up is carried out in a sterile liquid medium culture in an agitator and with controlled temperature for the appropriate period of time. When the growth period is completed, the purity of the culture is confirmed in agar plates by microscopic observation and Gram test; DNA fingerprinting procedures may also be performed (see de Bruijn, 2013). When that liquid culture is determined suitably, it is used to inoculate an industrial fermenter (Fig. 90.7).

Sampling of industrial fermenters is done daily and not only at the end of fermentation. In addition to the already mentioned controls, an estimation of microbial growth is carried out by spectrophotometry. For peat-based inoculants, the peat has to be previously neutralized, ground to 200 mesh, raked in polyethylene bags, and sterilized by Gamma radiation. Impregnation is made aseptically, puncturing the bag with a needle and then sealing the hole. For liquid inoculants, the broth is packaged aseptically (Fig. 90.8). The final inoculant product is subjected to quality control. However, it is highly recommended to have an additional quality control performed by the reference institution (RI).

90.1.5 MERCOSUR: Regulatory Framework and Quality Control

The achievements of the use of PGPR inoculants, mainly following field experimentation in the world has been summarized (Okon and Labandera-González, 1994; Helman et al., 2011).

PGPR research and inoculant development in MERCOSUR was initiated during the workshop on *Azospirillum* held at MGAP, Montevideo, August 1993. Its conclusions were published (Okon and Labandera-González, 1994). In 1998, all MERCOSUR countries signed "Recommendation 9/97, SGT 8 Agriculture, MERCOSUR/GMC/RES 28/98," which includes the obligation to register inoculants and to establish a "RI" for each country, as well as defining its responsibilities. Actually, each country has adapted its own regulations according to it (see Chapter 101).

Argentina has IMYZA INTA Castelar as its RI and the Secretary of Agriculture SENASA is in charge of registering inoculants. Az39 is the recommended *Azospirillum* strain to inoculate maize and wheat. In 2005, the network "Red de Control de Calidad de Inoculantes (REDCAI) (Inoculants

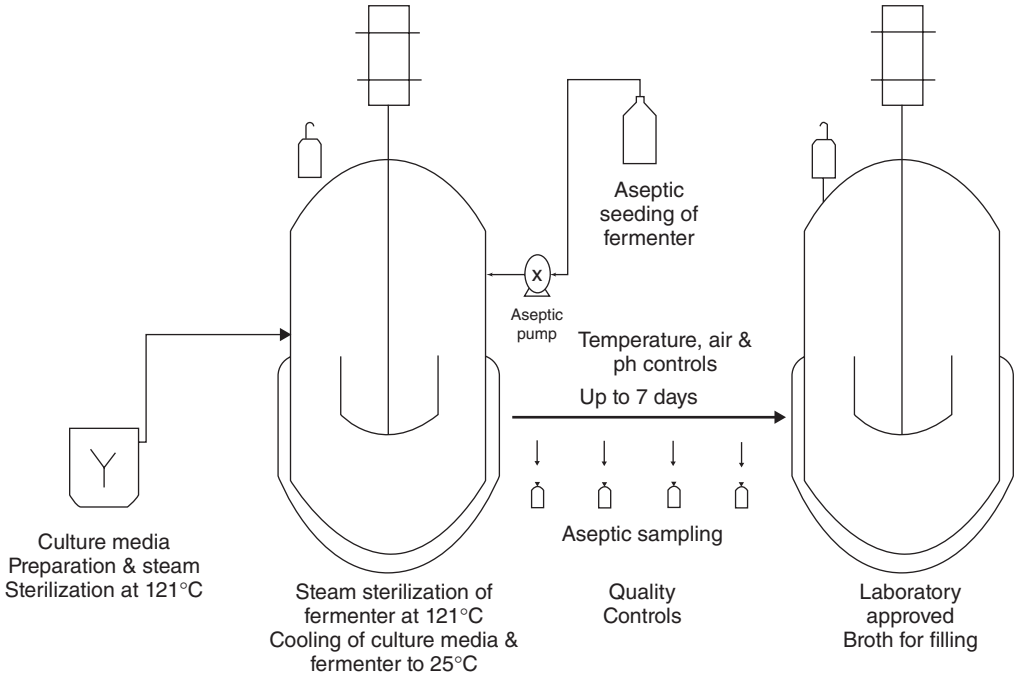

Figure 90.7 Industrial inoculant fermentation process.

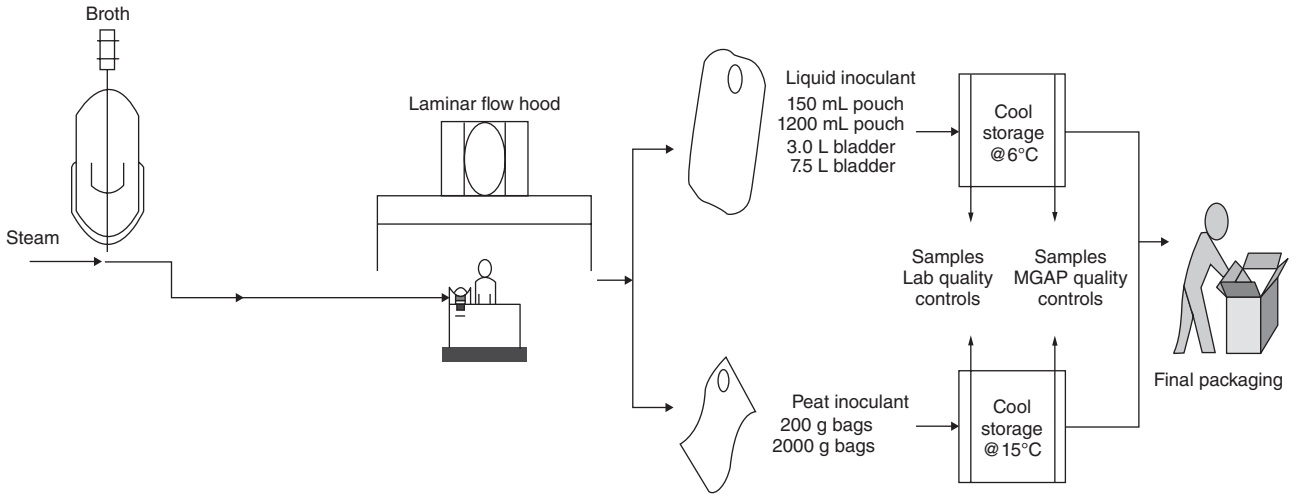

Figure 90.8 Inoculant aseptic filling and packing.

Quality Control Network)" was established as a member of the Division for Agricultural and Environmental Microbiology of the Argentinian Association of Microbiology, with the aim of creating and validating a set of methodological tools for the evaluation of inoculants (I Taller Iberoamericano REDCAI – RED BIOFAG, Asociación Argentina de Microbiología, Programa Iberoamericano de Ciencia y Tecnología para el Desarrollo, October 2010) (I Ibero-American Workshop REDCAI-REDBIOFAG, Argentinean Association of Microbiology, Ibero-American Science and TECHNOLOGY Program for Development). Procedure document 2 of REDCAI (Cassán et al., 2010) established the protocol for the quality control of *Azospirillum* inoculants.

Argentina is a pioneer in the development of PGPR inoculants and their field application. In the early 1980s, Silder Barrios and Enrique Rodríguez Cáceres began their work in obtaining several isolations of *Azospirillum* from diverse areas of their country. They formulated peat-charcoal based inoculants, which reached 84 days of shelf life in concentrations higher than 1×10^8 cfu/g (Puente et al., 2008). These researchers followed the guidelines of J. Döbereiner and Y. Okon to obtain several strains (Rodriguez Cáceres et al., 2008). The solid culture media "RC" was developed for isolation and identification of *Azospirillum* (Rodríguez Cáceres, 1982). Those strains were the initial material of the collection of the current Institute of Agricultural Microbiology and Zoology, IMYZA INTA Castelar. At the urging of Edgardo Muñoz Ratto, SENASA was the first institution of MERCOSUR to open registration for *Azospirillum*-based inoculants.

Proyecto Inocular emerged at the beginning of the last decade, led by Alejandro Perticari in a partnership between INTA and 25 inoculant companies, with the aim of promoting the technology of soybean inoculation, later extending it to other PGPRs, with more emphasis on *Azospirillum*. The local companies were the ones that started to develop *Azospirillum* inoculants (Table 90.1), with some of them that formulate mixtures of *Azospirillum* and other PGPR (Table 90.1). Laboratorios Alquimia was the first to launch a commercial product, Graminante. Its carrier is a wettable powder consisting of calcium carbonate and magnesium carbonate, registered as 20259 for maize and 20260 for wheat in 1992.

A few years later, work began simultaneously in Uruguay and Argentina with Graminosoil, a sterile peat-based inoculant, reaching high initial concentrations of *Azospirillum* per gram but with rapid death of the bacteria; authorization for open sale in Uruguay was obtained in 1992; and the registration number was 20284 for maize and 20285 for wheat in Argentina in 1994. Owing to difficulties in the use of peat-based inoculants in both countries, it was decided to change the carrier of Graminosoil to an aqueous media with at least 1×10^8 cfu/ml with 6 months shelf life.

Brazil has FEPAGRO as it RI. Strains Ab-V4, Ab-V5, Ab-V6, and Ab-V7 are recommended to inoculate maize and Ab-V1, Ab-V5, Ab-V6, and Ab-V8 for wheat (Hungria, 2011). The current recommendation for *Azospirillum* strains arises in part from the work carried out by Fabio Pedrosa at the Molecular Biochemistry and Biology Department of the Federal University of Paraná (Araujo, Solón, personal communication, March 2013). *Azospirillum*-based inoculants need to be registered at the Ministry of Agriculture, Livestock, and Food Supply (MAPA, in its Portuguese acronym) before being sold on the market and they must contain at least one of the recommended strains for each crop. Normative Instruction 30 sets the official methods for inoculant analysis. The Network of Laboratories for Recommendation, Standardization, and Dissemination of Microbial Inoculants of Agricultural Interest (Red de Laboratorios para Recomendación, Padronización y Difusión de Tecnología de Inoculantes Microbianos de Interés Agrícola - RELARE)

Table 90.1 List of Inoculant Products in the MERCOSUR

Company	Brand	*Azospirillum* Strains	Concentration at Expiry Date (cfu/ml-Liquid, cfu/g-Solid or WP)
Argentina			
AGRO FRANQUICIAS	AXION PLUS TRIGO	*Azospirillum* Az39	
AGRO INVEST	FULL BACTER	*Azospirillum* Az39	
ALQUIMIA	GRAMINANTE MAIZ	AZM3	5×10^4 WP
ALQUIMIA	GRAMINANTE TRIGO	AZT5	5×10^4 WP
ALTERBIO	ALTER PROMAZ TLS	*Azospirillum* spp.	1×10^7
ARBO	ENE 2 MAIZ	*Azospirillum* Az39	1×10^8
ARBO	ENE 2 TRIGO	*Azospirillum* Az39	1×10^8
AYUI	FACYT AZ	*Azospirillum* Az39	
BECKER UNDERWOOD	NITRAFIX	*Azospirillum* BR 1100	Origin of the inoculant-Brazil
BENEFICIAL GERMS	AZOGERMS	*Azospirillum* Az39	
BERANEK	NITROPLUS TRIGO		
BILAB	NITRO-FIX AZ ARROZ	*Azospirillum* Az39	2×10^8
BILAB	NITRO-FIX AZ MAIZ	*Azospirillum* Az39	2×10^8
BILAB	HOBER AZOS TRIGO	*Azospirillum* Az39	2×10^8
BILAB	HOBER AZOS MAIZ	*Azospirillum* Az39	2×10^8
BILAB	NITRO FIX AZ GIRASOL	*Azospirillum* Az39	2×10^8
BILAB	NITRO FIX AZ ALGODON	*Azospirillum* Az39	2×10^8
BIOTECH	N AZOSPIRRILLUM MAIZ	*Azospirillum* AbV5 + AbV6	1×10^7
BIOTECH	BIO NITROSEM MAIZ	*Azospirillum* spp.	1×10^7
CAMPOMAX	AZOMAX		
CERGEN	AZOSNITRO	*Azospirillum* Az39	
CHEMICAL – BIO	GRAMIBAC	*Azospirillum* spp.	1×10^8
CHEMICAL – BIO	RADIXIUS ONION	*Azospirillum* spp.	1×10^8
CKC	RHIZOFLO TRIGO	92078 ACTA 1390	1×10^6
CKC	RHIZOFLO GIRASOL	92079 ACTA 1390	1×10^6
CKC	RHIZOFLO MAIZ	*Azospirillum* Az39	1×10^6
ECOFERTIL	RAY GREEN TRIGO	*Azospirillum* ATCC 1003	1×10^8
ECOFERTIL	RAY GREEN MAIZ	*Azospirillum* ATCC 1003	1×10^8
ECOFERTIL	RAY GREEN GIRASOL	*Azospirillum* ATCC 1003	1×10^8
EMFAG	G3 AZUBAC	*Azospirillum* spp.	1×10^6
F.B.N.	F.B.N. TRIGO		
FARMCHEM	SOWER TRIGO		
FITOGENIA	AZOS-B		
FITOQUIMICA	FORTE	*Azospirillum* Az39	1×10^7
FPC ARGENTINA	AZP 2000	*Azospirillum* Az39	1×10^7
FPC ARGENTINA	AZP 2000	*Azospirillum* Az39	1×10^7
FRAGARIA	TRIGALAZO	*Azospirillum* Az39	1×10^8
FRAGARIA	GRAMINAZO PLUS	*Azospirillum* Az39	1×10^8
FRAGARIA	MAIZAZO	*Azospirillum* Az39	1×10^8
GREEN QUALITY	FOSTRIGON		
LANTHER QUIMICA	AZO LQ		
LOPEZ	NODUMAX	*Azospirillum* spp.	
MARKETING AGRICOLA	MARKETING AGRICOLA TRIGO	*Azospirillum* Az39	
MENAGRO	BIOCAMPO MAIZ		
MENAGRO	BIOCAMPO TRIGO		
NITRAP	AZOTRAP	*Azospirillum* Az39	
NITRAP	AZOTRAP PLUS – LETTUCE	*Azospirillum* Az39	
NITRASOIL	BIO-ENHACE	*Azospirillum* Az39	1×10^8
NITRASOIL	GRAMINOSOIL MAIZ	*Azospirillum* Az39	1×10^8

Table 90.1 (*Continued*)

Company	Brand	*Azospirillum* Strains	Concentration at Expiry Date (cfu/ml-Liquid, cfu/g-Solid or WP)
NITRASOIL	GRAMINOSOIL TRIGO	*Azospirillum* Az39	1×10^8
NIVELAGRO	NIVEL AZO		
NOVA	PROMOZION	*Azospirillum* Az39	
NOVA	INOCULANTE AGH		
NOVOZYMES BIOAG	NITRAGIN MAIZ	*Azospirillum* Az39	
NOVOZYMES BIOAG	NITRAGIN WAVE	*Azospirillum* Az39	
NOVOZYMES BIOAG	NITRAGIN SEMILLERO	*Azospirillum* Az39	
NOVOZYMES BIOAG	NITRAGIN NEW WAVE	*Azospirillum* Az39	
RAPARO, ANGEL	BUSCADOR N TRIGO		WP
RAPARO, ANGEL	BUSCADOR N MAIZ		WP
RAYBAC	RAYBAC		
RED SURCOS	DOMOX		
RIZOBACTER	RIZOSPIRILLUM	*Azospirillum*	
SAN PABLO	AZOLLUM H	*Azospirillum* TUC 27/85	
SAN PABLO	AZOLLUM TRIGO	*Azospirillum* TUC 27/85	
SAN PABLO	AZOLLUM MAIZ	*Azospirillum* TUC 27/85	
SAN PABLO	MACROMIX GIRASOL	Azopirillum TUC10/1	
SEMILLERA GUASCH	ZADPIRILLOM MAIZ	*Azospirillum* Az39	1×10^8
SEMILLERA GUASCH	ZADEN GRAMINEAS	*Azospirillum* Az39	1×10^8
SERV – QUIM	NITROFULL - G	*Azospirillum* spp.	
SINTESIS BIOLOGICA	BETERSEED	*Azospirillum* Az39	1×10^6
SINTESIS QUIMICA	NOCTIN AZO	*Azospirillum* Az39	1×10^8
SINTESIS QUIMICA	NOCTIN TURBA AZO	*Azospirillum* Az39	1×10^8(peat)
TRES E PRODUCTOS	AZZEA UNO		
WEIZUR	RIZOGROWTH AZP	*Azospirillum* Az39	
ZALAZAR, RODOLFO	LABZA LIQ AZO	*Azospirillum* Az39	1×10^7

Argentina-Continuation, Combined Inoculum

Company	Brand	PGPR #1	PGPR #2	Concentration
AGRO ADVANCE	PHOEBUS	*Azospirillum brasilense*	*Pseudomonas fluorescens*	
AGRO ADVANCE	PHOEBUS	*Azospirillum brasilense*	*Pseudomonas fluorescens*	
ALTERBIO	ALTER PSE		*Pseudomonas fluorescens*	1×10^8 cfu/ml
BILAB	NITRO FIX PF GIRASOL		*Pseudomonas fluorescens* BNM 233	1×10^7 cfu/ml
BILAB	NITRO FIX PF MAIZ		*Pseudomonas fluorescens* BNM 233	2×10^7 cfu/ml
BILAB	NITRO FIX PF TRIGO		*Pseudomonas fluorescens* BNM 233	1×10^7 cfu/ml
GREEN QUALITY	ECOFOS		*Pseudomonas* PS3	
BIAGRO	BIAGRO PSA LIQUID		*Pseudomonas aurantica*	1×10^7 cfu/ml
BIAGRO	BIAGRO PRO SOL		*Pseudomonas fluorescens*	1×10^7 cfu/ml
DEGSER	DEGFERTIL GIRASOL	*Azospirillum brasilense*	*Pseudomonas fluorescens*	
DEGSER	DEGFERTIL MAIZ	*Azospirillum brasilense*	*Pseudomonas fluorescens*	
DEGSER	DEGFERTIL TRIGO	*Azospirillum brasilense*	*Pseudomonas fluorescens*	
LIPHATECH LA	DESANGOSSE TRIGO		*Pseudomonas*	
LIPHATECH LA	DESANGOSSE MAIZ		*Pseudomonas*	
PALAVERSICH	BIOPOWER	*Azospirillum brasilense*	*Pseudomonas fluorescens*	
RIZOBACTER	RIZOFOS		*Pseudomonas sp.*	
RIZOBACTER	RIZOFOS-LIQ TRIGO		*Pseudomonas fluorescens*	1×10^9 cfu/ml
RIZOBACTER	RIZOFOS-LIQ MAIZ		*Pseudomonas*	1×10^9 cfu/ml

(*continued*)

Table 90.1 (*Continued*)

Brazil

Company	Brand	*Azospirillum strains*	Concentration at Expiry Date
GRUPO BIOSOJA	BIOMAX PREMIUM L	*Ab-V5*	2×10^8 cfu/ml
LABORATORIOS FARROUPILHA	AZOS		
NOVOZYMES BIOAG PRODUTOS	AZOMAX	*Ab-V5 + Ab-V6*	2×10^8 cfu/ml
SPRAYTEC	NODOFIX AZP MILHO/TRIGO		2×10^8 cfu/ml
STOLLER DO BRASIL	MASTERFIX GRAMINEAS	*Ab-V5 + Ab-V6*	2×10^8 cfu/ml
TOTAL BIOTECNOLOGIA	AZOTOTAL	*Ab-V5 + Ab-V6*	1×10^8 cfu/ml

Uruguay

Company	Brand	Origin	*Azospirillum* Strains	Concentration at Expiry date
CALISTER	BIOPROM AZ39	URUGUAY	*Azospirillum brasilense Az39*	1×10^9 cfu/ml
LAGE & CIA	GRAMINOSOIL	URUGUAY	*Azospirillum sp.*	1×10^8 cfu/ml
RUSPER	NITRAGIN MAIZ/WAVE	ARGENTINA *Az39*	*Azospirillum brasilense*	1×10^7 cfu/ml

Paraguay

Company	Brand	Origin	PGPR	Concentration at Expiry Date (cfu/ml-Liquid, cfu/g-Solid or WP)
AGROGANADERA PIRAPEY	AZP 2.000	ARGENTINA	*Azospirillum brasilense*	1×10^9 cfu/ml
AGROCONSULT	GRAMINANTE TRIGO	ARGENTINA	*Azospirillum brasilense*	5×10^4 cfu/g WP
AGROCONSULT	GRAMINANTE MAIZ	ARGENTINA	*Azospirillum brasilense*	5×10^4 cfu/g WP
BASF PARAGUAYA S.A.	NITRAGIN MAIZ	ARGENTINA	*Azospirillum brasilense*	1×10^9 cfu/ml
AGROLAND	RHIZOFLO LIQUID MAIZ	ARGENTINA	*Azospirillum brasilense*	1×10^7 cfu/ml
FRAGARIA DEL PARAGUAY	INOCULANTE GRAMINAZO	ARGENTINA	*Azospirillum brasilense*	2×10^9 cfu/ml
CHEMTEC	NUTRICHEM	PARAGUAY	*Azospirillum + Pseudomonas*	$5 \times 10^8 + 5 \times 10^8$ cfu/ml
BASF PARAGUAYA	NITRAGIN SEMILLERO	ARGENTINA	*Azospirillum brasilense*	1×10^8 cfu/ml WP
MATRISOJA	AZO TOTAL	BRAZIL	*Azospirillum brasilense*	2×10^8 cfu/ml
AGROFERTIL	GELFIX GRAMINEAS	BRAZIL	*Azospirillum BR 11005*	1×10^9 cfu/ml
ALQUIMICA	NOCTIN AZO	ARGENTINA	*Azospirillum brasilense*	1×10^9 cfu/ml
AGROLAND	RHIZOFLO PREMIUM	ARGENTINA	*Azospirillum + Pseudomonas*	$1 \times 10^9 + 1 \times 10^8$ cfu/ml
CAMPO FERTIL	BIOMAX PREMIUM MILHO	BRAZIL	*Azospirillum brasilense Ab-V5*	2×10^8 cfu/ml
DIAGRO	GRAMMY CROP	BRAZIL	*Azospirillum BR 11005*	1×10^9 cfu/ml
PLANAGRO	RADIMAX INOCULANTE	ARGENTINA	*Azospirillum brasilense*	1×10^9 cfu/ml

meets on a biannual basis, bringing together people from MAPA, EMBRAPA, Universities, ANPII (the inoculants chamber), and representatives from companies that are not associated to ANPII. Each RELARE may suggest that MAPA incorporate or discharge strains from FEPAGRO's recommendations. Table 90.1 summarizes the strains actually used by Brazilian companies.

Paraguay is a country that has received a large number of Argentinian and Brazilian farmers, who brought along with them their agricultural practices, among them, grain inoculation, and have created demand for the products they used in their own countries. NUTRICHEM, of CHEMTEC Company, is the first co-inoculant produced in Paraguay that contains *Azospirillum* and *Pseudomonas* (Table 90.1). Resolution 564 and its Annex regulate the registration of inoculants with Servicio Nacional de Calidad y Sanidad Vegetal y de Semillas (SENAVE; National Service for Plant and Seed Quality and Health) (Galeano Samaniego, M and Marecos, C, personal communication, 2013).

Uruguay appointed MGAP as its reference institution. More than 95% farmers inoculate their legume seeds. Adoption of this practice comes from a successful strategic country plan that started in the early 1950s, developed by MGAP with the objective to increase animal productivity through the

improvement of natural pastures by the use of phosphorous fertilizers and inoculated legumes focusing in the diffusion of the technology to reach farmers. *Rhizobium* legume inoculant production and its use are regulated in the country by Decrees MGAP 546/81 and MGAP 7/99 while other PGPR inoculants are by Resolution MGAP 03/2013. There are three *Azospirillum* inoculants on the market (Table 90.1) and one product, ENDO RICE, that contains a strain of *Herbaspirillum* isolated from local flood irrigated rice systems (Punschke and Mayans, 2011).

MERCOSUR may allow registration of an inoculant with strains that are not recommended, but the registrant company shall submit reports of efficacy under laboratory and greenhouse conditions, together with data from at least 3 years of field trials in three different agro-ecological zones.

90.1.6 Summary and Concluding Remarks

The experience achieved with *Rhizobium* inoculants in relation to storage, use, and management of inoculants and treated seeds (see also Chapter 97) has been transferred to PGPR products. Good inoculation procedures are important to achieve uniform distribution and high average load of bacteria on every seed. It is imperative for the efficiency of the process to use specifically designed inoculation machinery for each kind of seed. It is always recommended to follow the guidelines stated on the label. Every inoculant company should have information about compatibility of their product with chemical treatments used on the seeds, such as fungicides, insecticides, and bird repellents, as they may affect viability of bacteria on inoculated seeds. The timing between inoculant applications and sowing, as well as crop management (previous crop, type of sowing, seed density, fertilization, and irrigation) need to be planned for an optimal inoculant response.

It is important to point out that the degree of adoption and relative agronomic success of the use of PGPR inoculant technologies will depend on the quality of the products and their characteristics (powdered, peat based or liquid) and the way of application. Therefore, in order to ensure permanent adoption of the technology, it is necessary to

- have a coordinated multidisciplinary research institution to provide technical bases for inoculant formulation and application, such as carriers, strains, concentration, transportation and storage conditions, shelf period, recommendations, and precautions for use;
- have a regulatory framework setting high standards to assure inoculant quality in the market;
- develop a unique communication program to be transferred to agricultural engineers, extension agents, and farmers, based on local agronomic expected responses.

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Chapter 91

Auxin Signaling in *Azospirillum brasilense*: A Proteome Analysis

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91.1 INTRODUCTION

The auxin family is by far the best studied family of plant hormones. Still, major breakthroughs have been reported recently concerning the molecular mechanisms of auxin signaling, with the isolation of receptor, transport, and signal transduction proteins (Santner and Estelle, 2009; Zhao, 2010). Auxin synthesis is not restricted to plants. Diverse bacterial and fungal species are known to produce auxin, with indole-3-acetic acid (IAA) as the best documented auxin molecule (Spaepen et al., 2007). In the plant-growth promoting rhizobacterium (PGPR) *Azospirillum brasilense*, IAA production has been extensively studied and multiple biosynthesis pathways have been shown, with the pathway via indole-3-pyruvate (Trp → indole-3-pyruvate → indole-3-acetaldehyde → IAA), the most abundant one in the presence of the precursor tryptophan (Prinsen et al., 1993). In addition, more recent is the discovery of IAA as a signaling molecule in some bacteria and lower eukaryotes such as *Escherichia coli* (Bianco et al., 2006), *Agrobacterium tumefaciens* (Liu and Nester, 2006), and *Saccharomyces cerevisiae* (Prusty et al., 2004). Also in *A. brasilense*, IAA can influence gene expression. The key gene in IAA production (encoding an indole-3-pyruvate decarboxylase – *ipdC*) is upregulated by the end-product IAA (positive feedback regulation) (Vande Broek et al., 1999). Recently, we analyzed the transcriptional changes caused by auxin (exogenously added or endogenously altered by inactivation of the *ipdC* gene) for *A. brasilense* demonstrating that IAA is an important trigger for *A. brasilense* to enter into an interaction with plants (Van Puyvelde et al., 2011). To further investigate the signaling role of IAA in *A. brasilense*, a comparative proteome analysis of the wild-type strain and an auxin-impaired mutant (inactivation of *ipdC* gene) was conducted to identify differentially expressed proteins. Identification of these proteins can further unravel the complex role of IAA in the physiology of *Azospirillum*.

91.2 METHODS

The wild-type strains *A. brasilense* Sp245 (Baldani et al., 1986) and auxin-impaired mutant FAJ0009 (Costacurta et al., 1994) were grown in Luria–Bertani medium (per liter: 10 g tryptone, 10 g NaCl, 5 g yeast extract) supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ (L* medium). Samples for protein extraction were taken at early-exponential growth phase (OD_{595} 0.3–0.35). Protein purification, sample preparation, protein separation, and protein identification were carried out as described by De Mot and Vanderleyden (1989) and De Mot et al. (2007). In short, proteins were isolated by the phenol procedure and after precipitation solubilized in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% DTT, 2% IPG buffer pH 4–7 – GE Healthcare). Protein concentrations were determined using the PlusOne 2D Quant Kit (GE Healthcare). Separation of the isolated proteins was performed by isoelectric focusing using nonlinear Immobiline DryStrips (24 cm, pH 3–7; GE Healthcare). After rehydration of the strips, proteins (50–100 µg) were applied by cup loading. After focusing, the reduced strips were mounted on SDS-PAGE gels

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(12.5% polyacrylamide; $20 \times 24\,cm^2$), and the proteins were separated according to their molecular weight using an EttanDalt Six electrophoresis system (GE Healthcare). After separation and protein fixation, gels were stained overnight using Sypro Ruby (Invitrogen). After rinsing, gels were scanned with a Typhoon 9400 imager (GE Healthcare). The scanned gel images were analyzed using the ImageMaster 2D Platinum software (GE Healthcare). After normalization of the spot intensities, the ratio (threshold value of 1.3) between corresponding spots in the two conditions (wild-type versus mutant – three biological repeats per strain) was determined. These spots were cut from gels using an Ettan Spotpicker (GE Healthcare). For protein identification, the proteins were tryptic digested in-gel, and the eluted peptides were analyzed by quadrupole–time-of-flight (Q-TOF). Mass fingerprints were submitted to MASCOT database for identification using the genome sequence of *A. brasilense* Sp245 (Wisniewski-Dyé et al., 2011; see Chapter 25) as search database.

91.3 RESULTS AND DISCUSSION

The role of auxin biosynthesis in *A. brasilense* was further examined using a comparative proteome analysis. Therefore, the proteome map of cells harvested in the exponential phase of both the wild-type and auxin-impaired mutant was generated (see Fig. 91.1).

Comparative analysis of the spot intensities of the two proteome maps revealed about 15 proteins that could be identified to be differentially expressed in FAJ0009 compared to the expression level in *A. brasilense* Sp245. As FAJ0009 is a knock-out mutant in the key gene of IAA production, *ipdC*, and produces only 10% IAA of the wild-type level, we can speculate that these proteins are regulated directly or indirectly by IAA. Eight of these differentially expressed proteins could be confidently identified by Q-TOF analysis.

Two proteins were upregulated in FAJ0009 (see Table 91.1). The upregulation of the heat-shock protein DnaK is somehow surprising compared to the observations in IAA-treated *E. coli* cells: nontreated *E. coli* cells have a lower DnaK level than IAA-treated cells. It was suggested that IAA improves *E. coli*'s defense response against adverse conditions (Bianco et al., 2006). In *A. brasilense*, we observed the opposite effect since the DnaK level is higher in the low IAA background (i.e., in FAJ0009). The second identified protein, inosine-5′-monophosphate dehydrogenase GuaB, catalyzes the first step in the guanosine biosynthesis converting inosine-5'-monophosphate into xanthosine-5′-monophosphate. This protein has also been shown to be involved into stress resistance in the symbiotic bacterium *Rhizobium tropici* CIAT899. A *R. tropici guaB* mutant was unable to grow under elevated temperatures and was defective in symbiosis with common bean (Riccillo et al., 2000).

Six proteins were found to be downregulated in the mutant strain FAJ0009 (see Table 91.2). The indole-3-acetic acid biosynthesis controlling (IaaC) protein, identified as a repressor for IAA production (Vande Broek et al., 2005), is slightly downregulated, indicating that the mutant recues its auxin production by lowering the level of the IaaC protein.

A remarkable observation is the strong downregulation of the sugar-binding periplasmic protein SbpA. This protein was previously identified being upregulated (at both transcript and protein levels) by root exudates (Van Bastelaere et al., 1993, 1999). The protein is involved in the uptake of galactose and possibly functions in the chemotaxis of *A. brasilense* to the plant root (Van Bastelaere et al., 1999). The downregulation of SbpA in the mutant strain FAJ0009 indicates that auxin production is needed to have

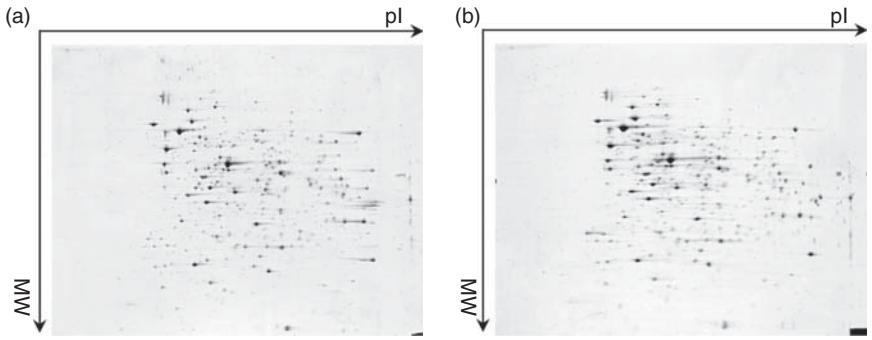

Figure 91.1 Two-dimensional PAGE analysis of proteins of *A. brasilense* and auxin-impaired mutant FAJ0009. Profile of proteins extracted from early-exponential phase (a) wild-type and (b) FAJ0009 cells. Proteins were separated according to pI (horizontally) and molecular weight (vertically) as described in the text.

Table 91.1 Proteins That are Upregulated in FAJ0009

Gene ID	Protein Name	Fold Induction
AZOBR_10493	Chaperone protein DnaK	1.38
AZOBR_p130122	Inosine-5′-monophosphate dehydrogenase	1.69

Table 91.2 Proteins that are downregulated in FAJ0009

Gene ID	Protein Name	Fold Repression
AZOBR_10236	Cysteine synthase	1.30
AZOBR_40353	Indole-3-acetic acid biosynthesis controlling protein	1.54
AZOBR_p280093	Extracellular solute-binding protein	1.55
AZOBR_40113	Cobaltochelatase small subunit	1.55
AZOBR_140167	Succinic semialdehyde dehydrogenase	2.14
AZOBR_p440163	Sugar-binding periplasmic protein SbpA	5.76

expression of certain parts of the chemotaxis system of *A. brasilense*. The *sbpA* gene was also identified to be upregulated by *N*-acyl-homoserine lactone (AHL)-dependent quorum-sensing in a proteome analysis of rice-associated *Azospirillum lipoferum* B518 (Boyer et al., 2008). However, it is difficult to link these results to our data, since AHLs are not synthesized by *A. brasilense* species (Vial et al., 2006).

Other downregulated proteins in FAJ0009 are cysteine synthase (or *O*-acetylserine(thiol)-lyase – CysK), involved in cysteine biosynthesis and tellurite resistance in *A. brasilense* Sp7 (Ramirez et al., 2006), an extracellular solute-binding protein, which is the periplasmic substrate-binding component of a putative ATP-binding cassette (ABC) transporter, and the small subunit CobS of cobaltochetalase, involved in the incorporation of the cobalt ion in the prosthetic tetrapyrrole group via the aerobic pathway for the synthesis of cobalamin or vitamin B_{12} (Holliday et al., 2007). As for GuaB, cobalamin biosynthesis is essential for symbiosis as demonstrated using cobalamin biosynthesis mutants for the *Sinorhizobium meliloti–Medicago sativa* and *Sinorhizobium fredii–Vigna radiata* symbiosis (Campbell et al., 2006; Medina et al., 2009).

The last identified downregulated protein in FAJ0009 is the succinic semialdehyde dehydrogenase GabD, catalyzing the conversion of succinate semialdehyde into succinate (which can enter the tricarboxylic acid cycle (TCA) cycle). It is involved in the metabolism of amino acids such as glutamate and tyrosine, but a role in bacteria–plant interactions has not yet been described.

The same conditions used in this proteome analysis (comparing wild-type and *ipdC* mutant cells from early-exponential phase) were already applied in a transcriptome analysis (Van Puyvelde et al., 2011). Therefore, we compared the differential expression data between the transcript and protein levels. However, for most differentially expressed proteins, no significant differential expression at the transcript level was observed. The only exception is the *iaaC* gene: a lower expression to the same extent as at the protein level was observed in the microarray analysis (1.50-fold repression in FAJ0009 – P value <0.001). Interestingly to note is the strong induction of the extracellular solute-binding protein AZOBR_p280093 at the transcript level, but this is only observed by the addition of external IAA.

In general, no major overlaps between expression at the transcript and protein level could be observed. This is not specific for this study: integration of "omics" studies is still challenging and not always straightforward (Kint et al., 2010).

91.4 CONCLUSION

Two-dimensional gel electrophoresis is a powerful high-throughput technique to analyze differentially expressed proteins. This technique was applied on *A. brasilense* to identify the role of IAA biosynthesis in the bacterial physiology. Proteome analysis of cells grown till early-exponential growth phase indicates that major differences can be observed at the level of protein expression between wild-type strain and the *ipdC* knock-out mutant FAJ0009. Nevertheless, it is difficult to pinpoint-specific processes associated with these changes: as for the changes at the transcriptional level, the identification of some differential proteins points toward a role in bacterial adaptation to stress and the plant environment.

Further experiments are planned to unravel differentially expressed proteins at other stages in the growth phase to further investigate the role of IAA in the bacterial physiology.

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Chapter 92

Genetic and Functional Characterization of *Paenibacillus riograndensis*: A Novel Plant Growth-Promoting Bacterium Isolated from Wheat

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92.1 INTRODUCTION

Ash et al. (1993) first described the genus *Paenibacillus* based on an analysis of the 16S rRNA gene sequence to accommodate the former "group 3" of the genus *Bacillus*. The name "paene" in Latin means almost, and therefore, the *Paenibacillus* is almost a *Bacillus*. The comparative 16S rRNA sequence analysis revealed that rRNA "group 3" bacilli represent a phylogenetically distinct group, exhibiting high intragroup sequence relatedness, and this species is only remotely related to *B. subtilis*, the canonical *Bacillus* species (Govindasamy et al., 2011).

Members of the genus *Paenibacillus* are facultative anaerobic organisms with G + C contents ranging from 45 to 54 mol% (Shida et al., 1997). Phenotypically, the species of this group react weakly to Gram's stain, and even young cultures appear gram-negative. This genus is composed of endospore-forming bacteria that differentiate into ellipsoidal spores, which distinctly swell the mother cell (Govindasamy et al., 2011).

Endospore-forming bacteria are essentially ubiquitous in agricultural systems. Common physiological traits important to their survival include the production of multilayered cell wall structures, the formation of stress-resistant endospores, and the secretion of peptide antibiotics, peptide signal molecules, and extracellular enzymes. However, significant variation exists in other key traits, including nutrient utilization, motility, and physicochemical growth optima (Gardener, 2004). Native populations of *Bacillus* and *Paenibacillus* occur abundantly in most rhizosphere soils and plant tissues and are differently colonized by distinct subpopulations (Mahaffee and Kloepper, 1997; Seldin et al., 1998).

Members of the *Paenibacillus* genus are widely distributed in nature and have diverse physiological characteristics. At present, there are 141 *Paenibacillus* species

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[http://www.bacterio.cict.fr/p/paenibacillus.html], including 21 species having nitrogen-fixing ability: *P. polymyxa* (Grau and Wilson, 1962), *P. macerans*, *P. durus*, and *P. peoriae* (Montefusco et al., 1993), *P. borealis* (Elo et al., 2001), *P. graminis* and *P. odorifer* (Berge et al., 2002), *P. brasilensis* (von der Weid et al., 2002), *P. massiliensis* (Roux and Raoult, 2004), *P. wynnii* (Rodríguez-Dias et al., 2005), *P. sabinae* (Ma et al., 2007a), *P. zanthoxyli* (Ma et al., 2007b), *P. donghaensis* (Choi et al., 2008), *P. forsythiae* (Ma and Chen, 2008), *P. sonchi* (Hong et al., 2009), *P. riograndensis* (Beneduzi et al., 2010), *P. sophorae* (Jin et al., 2011a), *P. jilunlii* (Jin et al., 2011b), *P. beijingensis* (Gao et al., 2012), *P. brassicae* (Gao et al., 1993), and *P. taohuashanense* (Xie et al., 2012). This genus harbors strains of industrial and agricultural importance. Some of these organisms excrete diverse assortments of extracellular polysaccharide enzymes to hydrolyze complex carbohydrates (Shida et al., 1997), such as glucanotransferase, chitinase, amylase, cellobiohydrolase, agarase, cellulose, and proteases (Aguilera et al., 2001; Sakiyama et al., 2001; Alvarez et al., 2006; Aktuganov et al., 2008; Moon et al., 2011). A number of *Paenibacillus* species also produce polysaccharides (Yoon et al., 2002) and antifungal and antimicrobial agents (Chung et al., 2000; Rosado and Seldin, 1993; Mavingui and Heulin, 1994; Walker et al., 1998; Sakiyama et al., 2001; Tupinambá et al., 2008).

The niche specificity and important ecological activities in *Paenibacillus* species span phylogenetic boundaries. Most species survive as saprophytes in soils, which are the primary reservoirs of these bacteria together with strains belonging to the *Bacillus* genus, and most viable cells exist as inactive spores at any given time (Nicholson, 2002). Furthermore, multiple species can be recovered as epiphytes and endophytes of plants and animals, and foodstuffs and composts are derived from these structures (Stahly et al., 1992; Slepecky and Hemphill, 1992). The rich variety of organic substrates and microniches present in these environments supports a complex milieu of microbial species; therefore, it is not surprising that multiple species of bacilli inhabit these niches (Gardener, 2004).

Numerous *Paenibacillus* species might contribute to the health status of plants in a variety of ways. A considerable number of isolates belonging to this genus have been used as agents of biological control against phytopathogens (Jung et al., 2003; von der Weid et al., 2005; Li et al., 2010; Tu et al., 2013). Yet, the successful application of these agents requires comprehensive knowledge of their ecology. In addition, the safety and efficacy of inoculants are largely determined by the ecological success of strains in the environments in which they are introduced. Better knowledge of the diversity, distribution, and action of bacilli is extremely important for the identification of new strains, the formulation of inoculants, and the determination of the types of plantation these bacteria might be associated with (Gardener, 2004).

Species of the *Paenibacillus* genus are typically beneficial free-living soil bacteria (Garbeva et al., 2003; Lee et al., 2007; Beneduzi et al., 2008a, 2008b) that influence plant growth and health, conferring one or more growth-promoting characteristics as plant growth-promoting rhizobacteria (PGPR) (Kloepper et al., 1989).

PGPRs indirectly or directly affect plant growth. The direct promotion of plant growth through PGPRs typically involves facilitating resource acquisition or modulating plant hormone levels (Glick, 2012). PGPRs can fix atmospheric nitrogen and supply it to plants, and some *Paenibacillus* species consistently show a great capacity to fix atmospheric nitrogen *in vitro* (Elo et al., 2001; Berge et al., 2002; Jin et al., 2011a). These species synthesize siderophores that solubilize and sequester iron from the soil and provide it to plant cells (Beneduzi et al., 2008a,b; Wen et al., 2011). Many strains synthesize several different phytohormones that stimulate various stages of plant growth (Çakmakçi et al., 2007; da Mota et al., 2008; Beneduzi et al., 2008a, 2008b; Lal and Tabacchioni, 2009) and might possess mechanisms for the solubilization of minerals, such as phosphorus (Hayat et al., 2010; Vazquez et al., 2000), that subsequently become more readily available for plant growth. A particular PGPR might affect plant growth and development through one or more of these mechanisms. The indirect promotion of plant growth occurs when PGPRs, acting as biocontrol bacteria, reduce the inhibitory effects of various pathogenic agents on plant growth and development (Glick, 2012). The direct antagonism of plant pathogens by *Paenibacillus* spp. has been well reported, and several strains can be used as biocontrol agents (von der Weid et al., 2005; Ryu et al., 2006; Senthilkumar et al., 2007; Zhou et al., 2008; and many others).

In recent years, *Paenibacillus* and *Bacillus* spp. have attracted considerable attention due to their advantages over other PGPR strains in inoculant formulations, stable maintenance in rhizospheric soil, and increased potential in sustainable agriculture (Govindasamy et al., 2011).

92.1.1 *Paenibacillus riograndensis*

Beneduzi et al. (2008b) investigated 311 strains of bacilli (primarily species of the genera *Bacillus* and *Paenibacillus*), displaying the important plant growth-promoting characteristics of isolates from seven distinct wheat production zones of the Rio Grande do Sul State, south Brazil. Of those isolates, SBR5, CSR16, SVPR30, and EsR7 were identified by the 16S rRNA gene sequences as strains of *Paenibacillus* sp. and selected for use in additional *in vivo* experiments in a greenhouse. The results showed that these species efficiently promoted a significant increase in the shoot and dry matter of wheat [*Triticum aestivum*] plants (Fig. 92.1).

The diazotrophic strain SBR5 was selected as a morphological, phylogenetic, and physiologically novel PGPR

Figure 92.1 The effect of inoculation of native PGPR isolates on wheat growth promotion, 30 days after sprouting. Plants were inoculated with CSR16 (1), SBR5 (2), SVPR30 (3), and EsR7 (4) strains; (5) plant was inoculated with *Paenibacillus polymyxa* ATCC 10343 strain; (6) positive control (plant was irrigated with mineral fertilizer solution); (7) negative control (plant was irrigated with distilled water).

bacterium, referred to as *Paenibacillus riograndensis* (ri.o.gran.den.sis. N. L. masc. adj. *riograndensis* referring to Rio Grande do Sul, the State located in southern Brazil, where the strain was isolated; Beneduzi et al., 2010).

The bacteria of strain SBR5^T are gram-variable, rod-shaped, sporulating and motile. The isolate produces ellipsoidal spores with a typical striped pattern. On GB medium, the colonies are circular, convex, white, and translucent and typically grow 1–2 mm in diameter within 24 h at 28 °C. The optimal growth temperature is 28 °C, and the optimal growth pH is 7. Strain SBR5^T displays PGPR characteristics, as it is able to fix nitrogen and to produce siderophores and indole-3-acetic acid (Beneduzi et al., 2010).

SBR5^T is phylogenetically related to members of the genus *Paenibacillus*, and the closest related species include *P. graminis* RSA19^T [98.1% similarity], *P. odorifer* TOD45^T [95.8%], and *P. borealis* KK19^T [96.3%]. The phylogenetic analysis based on *nifH* sequences revealed that the SBR5^T strain also clustered together with species of the genus *Paenibacillus* and showed high levels of *nifH* gene sequence similarity with *P. graminis* [78%], *P. wynnii* [79%], *P. odorifer* [77%], and *P. borealis* (74%] (Beneduzi et al., 2010).

Among the 814 genome sequences of Firmicutes listed in the Microbial Genomes of the National Center for Biotechnology Information, Paenibacillaceae comprise 51 genome projects, and the sequencing of the genome of the plant growth-promoting strain *Paenibacillus riograndensis* SBR5^T is now complete (Beneduzi et al., 2011). These data have been deposited in GenBank under the accession number DB SUB001272.

92.2 GENETIC AND FUNCTIONAL CHARACTERIZATION OF *Paenibacillus riograndensis*

92.2.1 Nitrogen-Fixing Genes

The great versatility of the secondary metabolism of many gram-positive bacteria makes these organisms suitable as biocontrol organisms against many different threats. From gram-positive bacteria, much is already known about human and animal pathogens. However, few studies have focused on beneficial plant-microbe interactions and plant growth-promoting gram-positive bacteria. For instance, the well-studied biological nitrogen fixation (BNF) has not yet been fully understood in gram-positive bacteria. The structural components for BNF have been identified and are primarily the same as those described in gram-negative bacteria, but the regulation of this process, which is crucial for enzyme functioning, has not been characterized for most of the gram-positive gene clusters. BNF is extremely costly, with respect to the energetic of this process, as 16 ATP molecules are used for each N_2 converted to ammonium, suggesting that tight regulation must occur (Dixon and Kahn, 2004).

Plant-associated gram-positive bacteria exist in two phyla: the Firmicutes and the Actinobacteria. *Frankia* spp. (Actinobacteria) are able to fix nitrogen in the free-living state and symbiotically with the non-legumes of a taxonomically diverse group of Angiosperms (Gtari et al., 2012; see Chapters 24, 35, 42, 43, 55, 75).

The typical regulation of nitrogen fixation for gram-positive bacteria is somehow different from that of gram-negative bacteria, potentially reflecting the lack of transcriptional control through the usual NifLA system present in gram-negative bacteria (see Chapters 9, 10, 11). However, in a recent study of *P. durus*, the authors showed that the presence of ammonium, even at high concentrations, did not inhibit the expression of *nifH* and *anfH*. For this bacterium, there are at least three copies of the *anfH* gene, although one of the copies is not transcribed (non-functional gene) (Teixeira et al., 2008). These authors suggest that, in the case of *P. durus,* regulation might occur at the post-translational level (see Chapter 13). Three copies of the *nifH* gene were also identified in *P. sabinae*, using RT-PCR (reverse transcription polymerase chain reaction) (Hong et al., 2012), and the expression of all copies was detected under

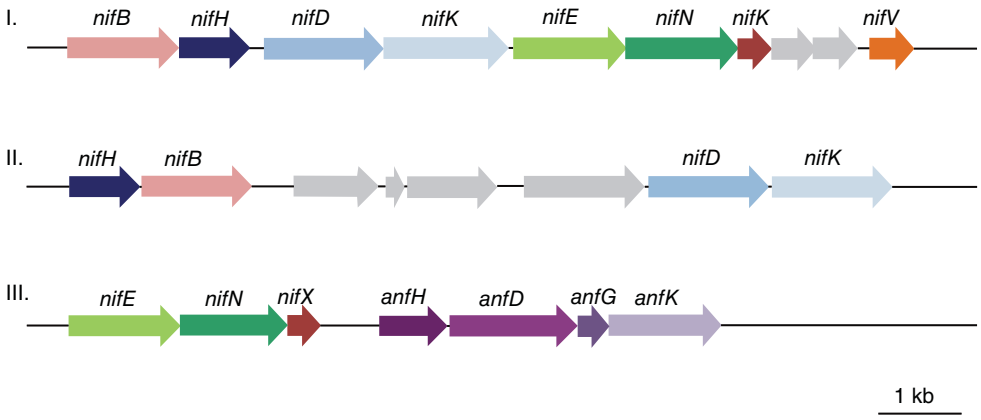

Figure 92.2 The genetic organization of the three clusters containing nitrogen fixation-related genes found in the *Paenibacillus riograndensis* genome.

nitrogen-fixation conditions. In the same study, the promoter regions of all *nifH* copies were tested in a heterologous system [*Escherichia coli* JM109], and the results indicated the activation of the promoters without the NifA activator, which is typically required for the activation of nitrogen fixation genes in gram-negative bacteria. A much different organization is identified in *Clostridium* diazotrophic species, such as *C. pasteurianum* (Kassap and Chen, 2005). In this bacterium, six copies of the *nifH* gene exist. Although the functionality of these copies has not been demonstrated, the transcription of the different copies can be detected using RT-PCR (Kassap and Chen, 2005).

In the plant growth-promoting bacterium *P. riograndensis, nif* operons have been described (Fig. 92.2): one complete operon containing *nifBHDKENXorf1orf2nifV* genes, located in a cluster; and an incomplete operon, with *nifHBorf1orf2orf3orf4DK* genes, and two copies of the *nifU* gene elsewhere in the genome. An *anf* operon was also identified in this genome, with *nifENXanfHDGK* genes.

The presence of an alternative nitrogenase gene cluster was also described for *P. azotofixans* (Rosado et al., 1998) and *C. pasteurianum* (Wang et al., 1988).

Preliminary studies for promoter characterization of the putative operons from nitrogen fixation genes in *P. riograndensis* have confirmed the lack of activation in the incomplete operon; however, activity of the *nif* and the *anf* operons has been reported. Using conventional nitrogen-limiting media, the bacterium makes use of the complete *nif* operon, activating Mo–Fe nitrogenase (G. Fernandes, personal communication). Similar to *P. durus* (Teixeira et al., 2008), the *anfH* gene and the entire promoter are also used in nitrogen fixation, as shown in the promoter tests with *P. riograndensis* (Fig. 92.3). Based on these results, experiments using culture media without the addition of molybdenum should demonstrate the increased promoter activity of the *anf* and *nif* operons.

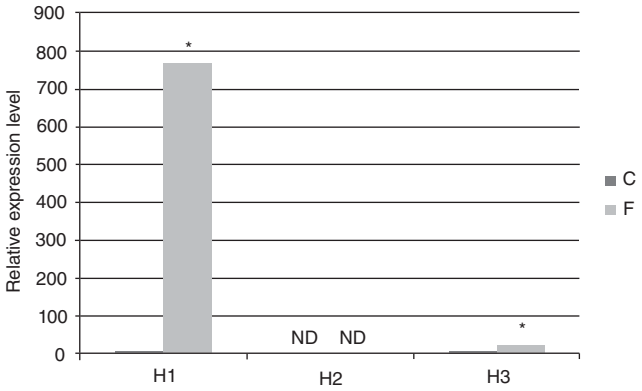

Figure 92.3 *Paenibacillus riograndensis* expression profile under nitrogen fixation conditions. The relative expression levels of the *P. riograndensis nifH1* (H1), *nifH2* (H2), and *anfH* (H3) genes (normalized to 16S rRNA expression) under standard nitrogen fixation conditions (F, cultivated on TBNR [N-free] medium) and control non-fixing conditions (C, cultivated on KB [N-rich] medium). ND: non-determined. * indicates statistically significant differences ($P < 0.05$, Student's T test) in comparison to control conditions.

92.2.2 Plant Growth-Promoting Characteristics

P. riograndensis was first isolated from wheat cultivated in the fields in south Brazil, in a study where bacteria were selected according to their ability to promote plant growth (Beneduzi et al., 2008b). The characteristics tested included nitrogen fixation, indolic compound and siderophore production, and phosphate solubilization, which showed a positive phenotype for this isolate (Beneduzi et al., 2008b). In the molecular studies of this bacterium, the *nifH* gene was amplified, and a phylogeny was reconstructed based on that sequence (see Chapters 3, 8, 20, 77). The phylogeny resulting from the NifH amino acid sequence was comparable to that of the 16S rDNA gene (Beneduzi et al., 2008b, 2010).

The phenotypical analysis and inoculation tests, using wheat as the host plant, confirmed the beneficial aspects of this bacterium toward the growth of this plant (see Fig. 92.1).

The isolate that generated the new species was able to produce a great amount of indolic compounds *in vitro* after 144 h of incubation (Beneduzi et al., 2008b). This important feature represents the direct effect of a PGPR to increase plant root growth and, in turn, to enhance plant nutrition. Other *Paenibacillus* species also show enhanced indolic compound production, such as *P. polymyxa* (Lebuhn et al., 1997; Quyet-Tien et al., 2008). The production of IAA (indolic-acetic-acid) from the tryptophan pathway (Quyet-Tien et al., 2008) has been previously demonstrated in *P. polymyxa*. In *P. riograndensis,* however, no genes with products involved in the tryptophan pathway were identified (Beneduzi et al., 2011). The exact mechanism used by this bacterium for the production of the plant hormone remains elusive.

Another important characteristic of a PGPR is the synthesis of siderophores, which chelate iron from soil and internalize the complex siderophore-Fe, providing iron in the soluble form for plants. The ability to grow in media when iron is unavailable was previously demonstrated in *P. riograndensis* (Beneduzi et al., 2008b). The type of siderophore produced or how it is transported in the bacterial cell is still not fully understood. However, copies of the genes *fhuCDB*, coding for siderophore recognition and uptake, were identified in the genome of *P. riograndensis*. There are four operons encoding these genes in this bacterial genome and two copies of the transcriptional regulator *fur*, which is the regulator of global iron metabolism (Beneduzi et al., 2011). Siderophore production has also been demonstrated in other species of the *Paenibacillus* genus. In the *P. elgii* strain, a gene cluster encoding the siderophore paenibactin was recently identified (Wen et al., 2011). *P. polymyxa* has previously demonstrated siderophore production, secreting small quantities of hydroxamate-type siderophores (Raza and Shen, 2010). These authors suggest that there might be a different mechanism for iron acquisition in this bacterium.

There is little information concerning the genetic process of phosphate solubilization in bacteria, although this phenotype is of great importance for a PGPR. Because many *Bacillus* species have demonstrated phosphate solubilization *in vitro* (Rodriguez et al., 2006), *Paenibacillus* species also present this feature. *P. riograndensis* was able to grow in media containing the insoluble form of phosphate complexed with calcium, suggesting that this bacterium possesses the ability to use mineral phosphate associated with hydrated oxides. The roots of plants associated with the bacterium can easily make use of the converted phosphate. A recent publication describes the solubilization of phosphates from the bacterium *P. polymyxa* under different phosphate sources (Wang et al., 2012).

Another important feature of PGPR is the production of antibiotic antagonistic substances. In the genome of *P. riograndensis,* genes with products related to antibiotic synthesis or transport were identified. Three different ORFs for the biosynthesis of monooxygenase antibiotic were annotated, and several genes with products related to the antibiotic tetracycline, such as efflux pumps, and membrane and resistance proteins, have been identified. Several other ORFs presenting products that function as resistance proteins for other antibiotics were also annotated. In other *Paenibacillus* species, the production of and resistance to antibiotics are also common. The recent release of a draft genome from *P. elgii* showed multiple sets of genes related to antibiotic biosynthetic pathways (Ding et al., 2011). Similarly, the existence of resistance genes has been described for several different strains of *P. polymyxa* (Jeong et al., 2011; Ma et al., 2011; Kim et al., 2010).

92.3 OTHER FEATURES OF *P. riograndensis* SPECIES

As with many bacillus, *P. riograndensis* has the capacity for sporulation (Beneduzi et al., 2010). Gram-positive bacteria use this feature as a survival strategy, according to the environment in which they are present. As soil bacteria, sporulation might confer an advantage when facing hostile conditions. Many genes related to this feature were identified in the *P. riograndensis* genome. Genes with functions related to germination, maturation, and spore structure have been identified in the *P. riograndensis* genome (Table 92.1). This feature was also investigated in *P. polymyxa* strains (Huo et al., 2010, 2012). In addition, the pathogenic species *P. larvae* presents remarkable endospore formation and has been the focus of recent research endeavors (Lindstrom et al., 2008; Alvarado et al., 2013).

Other interesting features for soil bacteria include chemotaxis and flagella formation. The genome of *P. riograndensis* presents a great number of ORFs related to these characteristics. Motility-related genes might provide an advantage to the bacterium in the soil, providing a mechanism to search for better conditions when the environment is not favorable. Another species of this genus that also displays chemotaxis-related motility is *P. vortex*. Many chemotaxis-related genes have been identified in the genome of this bacterium (Sirota-Madi et al., 2010). In *P. riograndensis*, the chemotaxis genes are located in the same cluster as the flagella formation genes.

The *P. riograndensis* bacterium possesses the important characteristics of a PGPR, and this potential was previously demonstrated *in vitro* through inoculation tests. In the future, this bacterium might be used in the formulation of an inoculant for wheat (*Triticum aestivum*), which is the natural host plant of this bacterium, and perhaps other grasses as well.

Table 92.1 Sporulation related ORFs identified in the *P. riograndensis* genome

ORF	Clusters of Orthologous Groups (COG) Classification	Product
0004.0005	D-Cell cycle control, cell division, chromosome partitioning	Spore cortex biosynthesis protein YabQ
0115.0001	R-General function prediction only	Spore maturation protein
0115.0002	R-General function prediction only	Spore maturation protein
0118.0005	R-General function prediction only	Lipoprotein LpqB, GerMN domain protein spore germination protein M
0207.0012	M-Cell wall/membrane/ envelope biogenesis	Spore coat-associated protein, camelysin
0212.0011	R-General function prediction only	GerA spore germination protein
0231.0035	R-General function prediction only	Spore germination protein
0231.0036	R-General function prediction only	GerA spore germination protein
0417.0018	M-Cell wall/membrane/ envelope biogenesis	Putative spore coat protein F
0417.0021	M-Cell wall/membrane/ envelope biogenesis	Putative spore coat protein F
0420.0009	M-Cell wall/membrane/ envelope biogenesis	Small acid-soluble spore protein alpha/beta type
0440.0004	M-Cell wall/membrane/ envelope biogenesis	Spore coat-associated protein JA
0579.0057	R-General function prediction only	GerA spore germination protein
0579.0060	R-General function prediction only	Spore germination protein

The full understanding of the microorganism is essential for the use of this bacterium in association with plants. The first step toward this goal was achieved after the sequencing of the genome of this bacterium, and based on the information obtained other gram-positive bacteria might also be used as PGPRs.

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Chapter 93

Role of *Herbaspirillum seropedicae* LPS in Plant Colonization

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93.1 LPS BIOSYNTHESIS MECHANISM IN *H. seropedicae*

Lipopolysaccharides (LPS) are outer membrane components of the gram-negative bacteria composed of three biosynthetically distinct structures: lipid A, core oligosaccharide, and O-antigen (Goldman and Hunt, 1990).

In most bacteria, the LPS biosynthesis genes are clustered with monosaccharide biosynthesis and modification genes, glycosyltransferases, sugar ABC-type transport systems, O-antigen ligase, polymerase, and other related genes (Reeves and Wang, 2006). The analysis of the *H. seropedicae* genome sequence (NC_014323) revealed the presence of several genes coding for proteins probably involved in LPS biosynthesis. These genes are dispersed in the genome, constituting small operons, a feature similar to that observed in *Gluconacetobacter diazotrophicus* (Bertalan et al., 2009).

Sequence analyses and the chemical structures elucidated by Serrato et al. (2012) helped suggest the biosynthetic pathway of the lipid-A portion of *H. seropedicae* LPS (Fig. 93.1). *H. seropedicae* LPS has five different fatty acids as constituents: 3OH-$C_{12:0}$ (54.6 mol% of LPS fatty acids), $C_{16:0}$ (29.4 mol%), 3OH-$C_{10:0}$ (11.0 mol%), $C_{12:0}$ (2.9 mol%), and $C_{14:0}$ (2.0 mol%) (Serrato et al., 2012). However, only the position of two N-substituted 3OH-$C_{12:0}$ in both β(1–6)-linked GlcN has been determined.

The O-antigen biosynthesis occurs through the transfer of UDP-sugars by glucosyltransferases to an acyl carrier undecaprenyl-PP to form the polymeric unit. *H. seropedicae* has 23 glucosyltransferase genes that could be responsible for those transfers. LPS saccharide constituents analysis showed five different monosaccharides: rhamnose (35.9 mol%), N-acetyl glucosamine (28.2 mol%), glucose (27.5 mol%), 3-deoxy-mano-oct-2-ulopiranosonic acid (6.2 mol%), and 4-N-arabinose (2.1 mol%) (Balsanelli et al., 2010). There are four described systems responsible for the O-antigen polymeric unit export and final assembly: Wzy/Wzx-, Wzm/Wzt-, synthase-, and Wzk-dependent systems (reviewed by Cuthbertson et al., 2010; Valvano, 2011). *H. seropedicae* gene content suggests that this bacterium uses an ATP-based transporter, the Wzm/Wzt-dependent system. In these systems, the polymeric O-antigen is formed on the cytoplasmic side of the plasma membrane prior to its export through an ABC-type transporter (Raetz and Whitfield, 2002; Valvano, 2003). After transport to the outer face of the plasma membrane, a specific glycosyltranferase called WaaL catalyzes a saccharyl transfer reaction from undecaprenyl-PP-O antigen to the lipid-A-core, forming the

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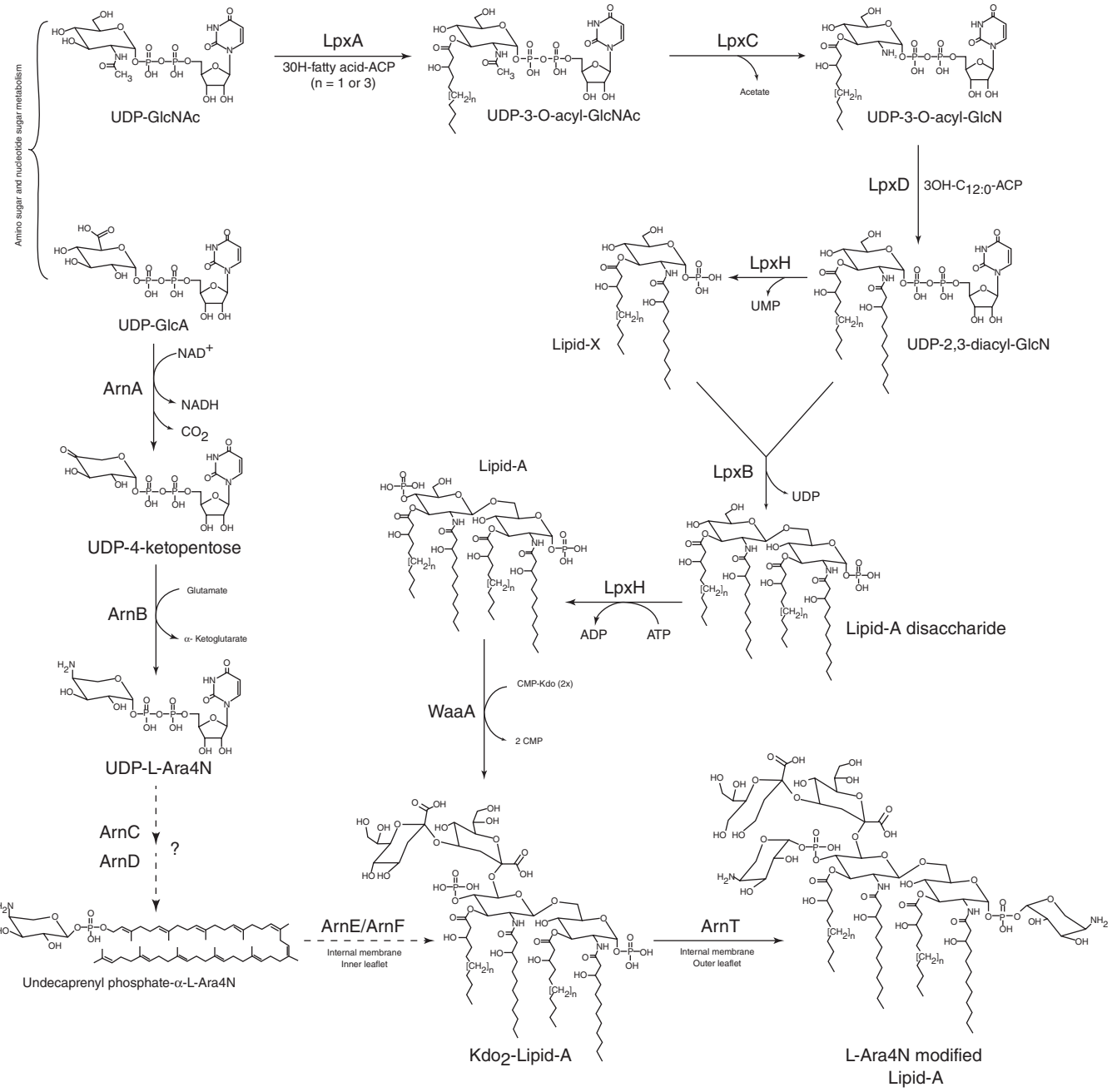

Figure 93.1 Biosynthesis pathway and structural model of the lipid-A portion of *H. seropedicae* LPS. LpxA (*Hsero_2190*) is a UDP-N-acetylglucosamine O-acyltransferase, LpxC (*Hsero_0343*) lipid-A-disaccharide synthase, LpxD (*Hsero_2188, 4206*) UDP-3-O-(3-hydroxymyristoyl) glucosamine N-acyltransferase, LpxH (*Hsero_1865*) UDP-2,3-diacylglucosamine hydrolase, LpxB (*Hsero_2191*) lipid-A-disaccharide synthase, WaaA (*Hsero_0475*) Kdo transferase, ArnA (*Hsero_2086*) UDP-glucuronic acid decarboxylase/UDP-4-amino-4-deoxy-L-arabinose formyltransferase, ArnB (*Hsero_2088*) UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate aminotransferase, ArnC (*Hsero_3963*) undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase, ArnD (*Hsero_4680*) 4-deoxy-4-formamido-L-arabinose-phosphoundecaprenol deformylase, ArnE (*Hsero_2089*) and ArnF (*Hsero_2091*) 4-amino-4-deoxy-L-arabinose-phosphoundecaprenol flippase subunit, and ArnT (*Hsero_0857, 2090*) 4-amino-4-deoxy-L-arabinose transferase.

complete LPS molecule. The LPS is then exposed to the outer membrane by a polysaccharide export protein (Wza) by a mechanism still to be known (Valvano, 2003, 2011).

93.2 LPS BIOSYNTHESIS REGULATION IN *H. seropedicae*

The expression of *H. seropedicae* LPS metabolism genes was tested using three *lacZ* fusions with genes involved in different levels of the biosynthesis. The first fusion evaluated the regulation of monosaccharide biosynthesis through analysis of the expression of the operon *rfbGgalErfbBC*, part of the rhamnose biosynthesis pathway, the major monosaccharide component of *H. seropedicae* LPS (Balsanelli et al., 2010). The second fusion evaluated the regulation of transport and assembly of lipid A-core portion through the analysis of expression of the operon *rfaLmsbAHsero3567rfaJJ2* (data not shown). The third fusion evaluated the regulation of assembly of the complete LPS molecule through the analysis of expression of the *waaL* (codes for O-antigen ligase) promoter (Balsanelli et al., 2013). The expression of the fusions was tested in the presence of maize plants, plant-derived compounds, antibiotics, and calcium ions.

Surprisingly, in the case of all the tested fusions calcium ions increased the expression of LPS metabolism genes. Addition of Ca^{+2} to *H. seropedicae* cultures led to a shift of polymerization level of O-antigen portion of the LPS, causing an increase in high molecular weight molecules (Balsanelli et al., 2010). Calcium is involved in several bacterial cellular processes, such as cell cycle and division, pathogenesis, motility, and chemotaxis (Michiels et al., 2002; Dominguez, 2004). Changes in intracellular Ca^{2+} concentration also alter the stability and activity of several enzymes, indicating that Ca^{2+} can act as a metabolic regulator (Rampersaud et al., 1991; Holland et al., 1999; Michiels et al., 2002; Dominguez, 2004). Indeed, Ca^{2+} plays an important role as a factor of host recognition and specificity in many bacteria (Economou et al., 1990; Ehrhardt et al., 1996; Gehring et al., 1997; Felle et al., 1998; Broughton et al., 2006). Under natural conditions, *H. seropedicae* could access calcium ions from the plant apoplast, where their concentration varies from 1 to 10 mM (Bush, 1995; Lecourieux et al., 2006), suggesting that transcription of LPS genes is upregulated in the bacterium when it enters living plant tissues. The mechanism of Ca^{2+}-dependent transcription regulation in *H. seropedicae* needs further investigation.

Polymyxin B sulfate and salicylic acid (SA) also alter the expression of the *rfb* operon, suggesting that the biosynthesis of LPS may be triggered by chemically aggressive compounds. The response of bacteria to SA through differential gene expression may be important to guarantee their survival upon entering the plant. For example, SA was shown to downregulate fitness and virulence factor production in *Pseudomonas aeruginosa* PA14 (Prithiviraj et al., 2005). In *Agrobacterium tumefaciens,* SA inhibited the induction of virulence genes (Yuan et al., 2006).

Some plant signals, such as flavonoids (see Chapter 50), might signal to the bacteria the presence of the plant host and induce expression of genes necessary for colonization, including the LPS genes. The plant flavonoid naringenin increased the expression of the *rfb* operon and *waaL*. Tadra-Sfeir et al. (2011) showed that *H. seropedicae* responds to naringenin by modifying the cell wall through altered expression of genes involved in outer membrane, cell wall, and peptidoglycan metabolism. Changes in the cell surface are documented in other bacteria that interact with plants, and probably such changes play a role in the interaction between the endophyte *H. seropedicae* and its host plants (Broughton et al., 2006; Quéré et al., 2006; Staehelin et al., 2006).

The expression of the three fusions was evaluated during the early interaction with maize. In addition to the fact that the presence of the plant increased LPS genes expression of surrounding free-living bacteria, a remarkable induction was observed in the cells attached to the maize root surface. Such induction suggests a role for the LPS as an anchoring molecule to the host.

In summary, *H. seropedicae* LPS synthesis is induced during interaction with maize by plant signals, indicating the importance of this molecule during host colonization.

93.3 LPS AS A BACTERIAL PROTECTION BARRIER

The bacterial surface makes the first contact with host cells and the host chemical defenses. Among several surface polysaccharides, the LPS of gram-negative bacteria is thought to be a protective wall, rendering bacteria resistant to a variety of plant defense molecules (Nikaido, 1994; Erbs and Newman, 2003). This protection barrier function is stressed by the fact that LPS allows the traverse of hydrophobic molecules at about 1–2% of the rates observed with typical phospholipid bilayers (Plesiat and Nikaido, 1992; Allende and McIntosh, 2003). Indeed, LPS has two potential ways to act as barrier: the packed saccharide portion of the molecule that forms a hydrophilic wall (Snyder et al., 1999), and the fatty acid portion of the lipid-A that constitutes a hydrophobic wall (Brandenburg et al., 1997; Andra et al., 2004). Several transcriptome analyses showed that the expression of cell envelope biosynthesis genes is modified during interaction with the host (D'Haeze and Holsters, 2004), indicating changes in the capsule structure. Such modifications can allow the bacteria to mimic the host immune system and suppress or evade immune reactions, to counteract the plant defense and proceed with the establishment of the interaction (Lerouge and Vanderleyden, 2002).

Changes in LPS structure often result in increased sensitivity to antimicrobial peptides, detergents, and other chemical stresses (Campbell et al., 2002; Lerouge and Vanderleyden, 2002; Jofré et al., 2004), such as sodium dodecyl sulfate (SDS), polymyxin B, and plant-derived flavonoids and SA. SA, a plant phenolic secondary metabolite, is a key regulator of plant defenses in response to a wide variety of pathogens (Martinez-Abarca et al., 1998; Prithiviraj et al., 2005; Stacey et al., 2006). During infection, SA, apart from acting as a bactericidal agent, triggers plant long-lived resistance to pathogens by either a localized or a systemic acquired response (Durrant and Dong, 2004; Prithiviraj et al., 2005). *H. seropedicae* is resistant to low concentrations of SA (up to 25 μg/ml) (Balsanelli et al., 2010), similar to the concentrations accumulated in bacterial infection sites of plants (16–23 μg/ml) (Huang et al., 2006). Alterations in LPS structure decreased this resistance, suggesting that the *H. seropedicae* LPS acted as a barrier to SA, preventing it from entering into the bacteria. This resistance to SA may be important to guarantee bacterial survival upon entering the plant, ensuring endophytic colonization. In fact, *H. seropedicae* mutants that display LPS alterations showed decreased endophytic colonization compared to the wild type (Balsanelli et al., 2010; 2013).

93.4 LPS AS AN ANCHORING SITE FOR BINDING ROOT LECTINS

Apart from the general function of protection barrier displayed by LPS molecules, a much more specific role for these molecules was described during attachment of *H. seropedicae* to the maize root surface.

Maize inoculation assays showed that efficient attachment of *H. seropedicae* requires the O-antigen of LPS, since the number of wild-type bacteria attached to maize root surfaces was approximately 100-fold higher than that of the mutant strains lacking the O-antigen. The possible role of bacterial outer membrane proteins in the attachment step was discarded because proteinase-K-treated bacteria attached normally on the roots. Also, the SDS-PAGE profile of outer membrane protein extract from LPS mutants was not different from that of the wild type, indicating the involvement of LPS O-antigen in attachment. On the other hand, when the roots were treated with proteinase-K, wild type attachment decreased to similar levels of the LPS mutants, suggesting that maize root proteins could act as receptors for bacterial attachment. The attachment capacity of the wild type strain was also reduced to a level equivalent to that of the LPS mutants when the assay was performed in the presence of isolated wild type LPS, glucosamine, or N-acetyl glucosamine, suggesting that these compounds blocked bacterial attachment sites. These data suggest that *H. seropedicae* LPS O-antigen participates in the attachment step of colonization by anchoring the bacterium to plant lectins through its N-acetyl glucosamine residues. Wheat germ agglutinin (WGA), a lectin with high affinity toward N-acetyl glucosamine, is present on surfaces of wheat seedlings and on root tips of adult wheat plants (Mishkind et al., 1983), and may therefore be a site for specific attachment of rhizobacteria contributing to bacterial adhesion to the root surface (Del Gallo et al., 1989). The WGA lectin was able to agglutinate *H. seropedicae* wild type cells, but not the *waaL* mutant (lacks the LPS O-antigen), in a concentration-dependent manner, indicating that the lectin is capable of binding to the *H. seropedicae* cell surface. WGA also inhibited attachment of wild type *H. seropedicae* to maize and wheat plants, but not that of LPS mutant, further supporting the LPS–lectin binding model for attachment.

Three lectin-like proteins from maize roots (MRLs - *M*aize *R*oot *L*ectins) were purified by affinity chromatography on an N-acetyl glucosamine-agarose column, since this monosaccharide inhibits *H. seropedicae* attachment to maize roots. Mass spectrometry analysis showed that proteins MRL-1 and MRL-2 are similar to uncharacterized jasmonate-induced maize proteins. Both proteins have a jacalin-like domain with six sugar binding sites, which may bind mono- or oligosaccharides with high specificity (Raval et al., 2004). MRL-3 contains a B chain domain, a two-domain lectin with aromatic residues that can interact with the nonpolar face of saccharides and polar residues for hydrogen bond formation to the sugar hydroxyl groups (Boraston et al., 2004). These proteins showed agglutination activity against wild type *H. seropedicae*, but failed to agglutinate the *waaL* mutant strain. The agglutination reaction was severely diminished in the presence of N-acetyl glucosamine. Moreover, addition of the MRL proteins as competitors in *H. seropedicae* attachment assays decreased the adhesion of the wild type to maize roots 80-fold. The results suggest that N-acetyl glucosamine residues of the LPS O-antigen bind to the MRLs, an essential step for efficient bacterial attachment and colonization.

The maize GlcNAc-binding lectins have homologs in other grasses such as wheat, rice, and sorghum, plants that are highly colonized by *H. seropedicae*. Indeed, lectins of several cereals, such as wheat, rye, barley, and rice, are related and can be inhibited by GlcNAc and its oligomers (Stinissen et al., 1985), suggesting that *H. seropedicae* may attach onto root surface in a similar manner in those hosts.

Azospirillum brasilense also interacts with WGA, although it is not clear which outer membrane polysaccharide is involved with this binding (Konnova et al., 1994). Co-inoculation of *H. seropedicae* and *A. brasilense* on maize plantlets showed that different bacterial species containing N-acetyl glucosamine in their outer layer may compete to colonize maize roots (and probably other grasses), and that the structure of such polysaccharides may contribute in determining the specificity of plant–bacteria interactions.

During competition, attachment of both species is reduced when compared to separated inoculation, probably due to sharing a conserved mechanism to bind to GlcNAc-lectins. The question of how exactly such plant proteins promote the binding of rhizobacteria on the root remains unsolved. Some lectins present membrane domains (including MRL-3) that could anchor them on the plant cell membrane, and consequently anchor the bacteria. However, several lectins seem to be secreted in the rhizosphere, agglutinating bacteria in the root mucilage or trapping them in the plant cell wall.

In summary, *H. seropedicae* uses the LPS as anchoring molecules that bind to root NAcGlc-lectins, enhancing the efficiency of bacterial attachment that confers a competitive advantage in the rhizosphere.

93.5 CONCLUSION

The LPS biosynthesis mechanism in *H. seropedicae* is highly conserved among gram-negative bacteria, despite the fact that the genes are dispersed in the genome. The regulation of expression of those genes in *H. seropedicae* seems to be unique, being dependent on plant signals and calcium levels, indicating the important role of LPS in the interaction with the host. The interaction between LPS O-antigen and the MRLs is a key step for the establishment of the bacteria onto the root, enhancing *H. seropedicae* attachment and conferring a competitive advantage during colonization of root surfaces. Such advantage may indicate a preferential access to the inner tissues. During colonization of these inner tissues, a complete LPS molecule appears to be necessary for resistance to plant basal defenses.

Associative bacteria such as *H. seropedicae* display molecular factors that facilitate the adhesion and colonization of plant surfaces and then the invasion of inner tissues. The expression of these molecular factors, including LPS, appears to be under the control of signals sent by the plant. This complex molecular communication between *Herbaspirillum* and plant hosts ensures an efficient association, which results in the promotion of plant growth and increased productivity. Interestingly, the already described molecular mechanisms used by *H. seropedicae* to interact with hosts share similarity to that described for some pathogenic bacteria, which may allow the bacteria to efficiently colonize diverse crops. LPS is a foremost and important factor of *H. seropedicae* for plant colonization, but other factors present in its genome are also required for the interaction. Future work, especially by using omics approaches such as the transcriptomic profile of the bacteria during attachment to maize roots, will identify new molecular players of this interaction and allow improving the model for the establishment of *H. seropedicae*–poacea association.

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Chapter 94

Culture-Independent Assessment of Diazotrophic Bacteria in Sugarcane and Isolation of *Bradyrhizobium* spp. from Field-Grown Sugarcane Plants Using Legume Trap Plants

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94.1 INTRODUCTION

The development and extension of biological based economy, the so-called "sustainable bioeconomy," is a major focus of scientific research and technological development in many countries, including Germany and Brazil. The German government recently launched a 6 billion Euro developmental program to develop a biological based economy. In Brazil, an important focus on development and support is on bioenergy crops such as sugarcane, which have ideal climatic conditions for high biomass development with high sugar content. The global goal for energy crops is to

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reduce the dependence on fossil energy sources – which are limited, which increase the price, and which, most importantly, cause major climatic problems for Planet Earth. The challenge is to grow energy crops, such as sugarcane, with the lowest inputs of fossil energy that is required to drive the industrial Haber–Bosch process of reducing atmospheric dinitrogen to ammonia, the basis for plant nitrogen fertilizer. In Brazil in particular, sugarcane plays a fundamental role in the production of bioethanol, which is used as a substitute for fossil fuels in combustion engines. In 2010, almost 10 million ha of sugarcane was planted in Brazil (IBGE, 2010) and approximately half of the cane juice has been fermented to produce alcohol as a biofuel. In contrast to many other energy crop systems, the overall energy balance in Brazilian sugarcane/bioethanol production is clearly positive (Boddey et al., 2008). For most other bioenergy processes, the overall energy balance is around one or even below. Since the energy to be spent for the synthesis of industrial nitrogen fertilizer is a major input, the reduction of fertilizer application would make the energy production even more attractive and efficient in terms of global climate change impact.

The biological nitrogen fixation (BNF) potential of sugarcane with its associated bacteria had come into focus already some decades ago (Döbereiner, 1961; Ruschel et al., 1975). Using ^{15}N-isotope enrichment tracing and natural abundance techniques, it was demonstrated that some sugarcane cultivars could derive up to 70% of plant nitrogen from BNF (Lima et al., 1987; Urquiaga et al., 1992; Boddey et al., 2001; see Chapter 108). A major breakthrough in the characterization of the diazotrophic bacteria, especially in plant associations, was the use of nitrogen-free semisolid media, such as NfB, JNfB, or LGI (Döbereiner, 1995), to efficiently enrich microaerobic nitrogen-fixing bacteria. This resulted in the characterization of many new diazotrophic species, such as *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Burkholderia tropica*, *Azospirillum amazonense,* and others (Cavalcante and Döbereiner, 1988; Reis et al., 2004; Perin et al., 2006; Baldani and Baldani, 2005; see Chapter 88). However, it could not yet be determined which of these bacteria contributed to the plant nitrogen fixation. It became clear that a diversity of diazotrophic bacteria are distributed in the whole plant, colonizing roots, stem, and leaves endophytically (James et al., 1994). Inoculation experiments with single or mixtures of diazotrophic bacteria were performed, which finally led to the development of the five-strain inoculum introduced by Embrapa Agrobiologia, Seropédica (Oliveira et al., 2003; Oliveira et al., 2006; Oliveira et al., 2009). These inoculations are presently being performed at different places in Brazil. Significant plant growth promotion effects have been found in production-scale field trials, but the level of BNF could not be increased significantly (Schultz et al., 2012). It also became apparent that the diversity of diazotrophic bacterial community of sugarcane plants is quite variable in different regions and cultivars (Asis et al., 2000; Asis et al., 2002; Magnani et al., 2010; Beneduzi et al., 2013; Suman et al., 2001). While a diversity of *Gluconacetobacter diazotrophicus* was found in many studies on different continents using LGI-semisolid media as an enrichment approach, in some other trials it was almost completely missing. In general, within the bacterial diversity retrieved from sugarcane by regular cultivation techniques without enrichment for diazotrophs, the diazotroph community was only found as a minor fraction (Magnani et al., 2010).

An alternative to the cultivation approach is the application of cultivation-independent molecular approaches, using 16S rDNA and *nifH*- or *nifD*-DNA probes, (see Chapters 3, 8, 20, 77) which have the advantage of covering also organisms that are slow growing or generally difficult to grow in pure culture. Several other plants have been investigated in detail with this approach, such as potato, maize, or rice (Roesch et al., 2006; Sessitsch et al., 2002, 2012), which has led to new insights in the diversity of diazotrophs in these systems and the overall bacterial community. Concerning sugarcane, Ando et al. (2005) retrieved for the first time *nifH*-sequences from sugarcane. More recently, Thaweenut et al. (2011), Burbano et al. (2011), and Fischer et al. (2012) studied *nifH*-transcripts in different sugarcane cultivars under different growth conditions. They found, besides *nifH*-sequences of other diazotrophs, also *nif*-transcripts of *Bradyrhizobium* and *Rhizobium* spp. In this chapter, the characterization of the diversity of active bacteria (16S cDNA) and active diazotrophs (*nifH* cDNA) in the sugarcane cultivar RB867515 is described, and the cultivation of *Bradyrhizbium* spp. from this plant using the legume trap plant *Vigna unguiculate* (cowpea) and direct cultivation on plates is presented.

94.2 MATERIALS AND METHODS

94.2.1 Greenhouse and Field Experiments

Greenhouse and field experiments were conducted at the Embrapa Agrobiologia CNPAB institute and experimental field station (22°45′S, 43°40′W and 26 m above sea level) in Seropédica, Rio de Janeiro, Brazil. The soil (Itaguai series) is classified as a Planosol (FAO) or Typic Fragiaquult (USDA, Soil Taxonomy). The soil characteristics in the 0–20 cm layer were pH (in H_2O) 5.4, 1.1 $cmol_c$ Ca^{2+}/dm^3; 0.2 $cmol_c$ Mg^{2+}/dm^3; 0.1 $cmol_c$ AL^{3+}/dm^3; 26.1 mg P/dm^3; 27.0 mg K/dm^3; 0.48% organic carbon, 0.83% organic matter, and 0.043% N. In the greenhouse experiment the cultivar RB867515 was tested in a Sand-Teosint Mix with six plants per pot; after 4 weeks the plants were harvested.

In the field experiments, the plants (cultivar RB867515) were grown for 2 years; the sugarcane plants were analyzed in late summer (March) of the first and second year. While there was no N-fertilization in the greenhouse experiment, there were plots in the field experiments that received urea (120 kg/ha). When the sugarcane plants were inoculated with the five-strain Embrapa-inoculum, the plots received no N-fertilization; more details are presented in Fischer et al. (2012).

94.2.2 DNA/RNA Extraction and PCR Approaches Characterizing the Bacterial Diversity *in planta*

RNA and DNA extractions of plant material were performed using the phenol–chloroform extraction method combined with the column-based AllPrep DNA/RNA Mini Kit (Qiagen, Germany) to purify and isolate RNA and DNA from 400 mg plant material as described by Toewe et al. (2011). Contaminating DNA in the RNA extractions was detected using a 16S rRNA coding gene targeted PCR (polymerase chain reaction) using the primers 341F and 907R (Muyzer et al., 1996). Contaminating DNA was removed using RQ1 RNA-free DNase (Promega, USA). An important step in this reverse transcription polymerase chain reaction (RT-PCR) protocol was that the cDNA was synthesized with the Omniscript RT Kit (Qiagen, Germany) using random primers (Promega, USA) to achieve a mostly unbiased reverse transcription. All PCR reactions were performed using the TopTaq Kit (Qiagen, Germany) in a PEQStar 96 Universal thermocycler (Peqlab, Germany). 16S rRNA cDNA was amplified using the primers 341F and 907R (length about 550 bp) (Muyzer et al., 1996). The annealing temperature for the PCR reaction was 56.5 °C and had 30 cycles. *nifH* mRNA was amplified using nested PCR with the primers PolF and PolR for the initial amplification (Poly et al., 2001) and *nifH*For and *nifH*Rev (length about 314–317 bp) for the second amplification step as described in Roesch et al. (2006). In the second PCR, the annealing temperature was 55 °C, and 33 cycles were used. Amplicons were verified using agarose gel electrophoresis. Genomic DNA from pure cultures of *Herbaspirillum seropedicae* HCC103, *H. rubrisubalbicans* HRC54, *Azospirillum amazonense* CBAmC, *Burkholderia tropica* Ppe8, and *Gluconacetobacter diazotrophicus* PAL5 (diazotrophs in the inoculation mix) was used as positive *nifH*-amplification controls using this nested PCR approach.

Amplicons of the 16S- and *nifH*-cDNA were purified using NucleoSpin® Extract II Kit (Macherey-Nagel, Germany) and cloned using Topo TA Cloning® Kit (Invitrogen, USA) according to the manufacturer′s instructions. Sequencing was performed using BigDye® Terminator (BDT) v3.1 Sequencing Kit (Invitrogen, USA) on an ABI 3730 sequencer (Applied Biosystems, Germany). See details of sequence analysis in Fischer et al. (2012). Evolutionary relationships in the phylogenetic tree were conducted using ARB software (Ludwig et al., 2004) with a neighbor-joining algorithm (Saitou and Nei, 1987).

94.2.3 Preparations for the Isolation of Endophytic Bacteria from Sugarcane

Unlignified white shoot roots from 5-month-old sugarcane plants (cv. RB867515) growing at the Embrapa Agrobiologia experimental field station were collected in June 2012. The roots were thoroughly washed with tap water and surface-disinfected by sequential immersion in 70% ethanol for 30 s, and in undiluted commercial bleach (2.5% hypochlorite) under gentle agitation for 8 min. Then the roots were washed six times with sterile water and the disinfection procedure was evaluated by plating 100 µl of the last washing water on plates containing yeast mannitol agar (YMA) (Vincent, 1970). In addition, the disinfected roots were placed on YMA plates for 10 s before continuing the isolation procedure. These plates were incubated for at least 10 days to permit bacterial growth.

The disinfected roots (1.8 g) were macerated in a sterile mortar and suspended in 17 ml saline solution (NaCl 0.85%). Seeds of *V. unguiculata* (cowpea) were surface-disinfected by immersion in ethanol (70%) and then in undiluted commercial bleach for 5 min. After washing six times with sterile water, the seeds were germinated on water agar (0.8%) for 3 days at 28 °C in the dark.

94.2.4 Isolation of Rhizobia from Sugarcane Tissues Using In Vitro Grown *V. unguiculata* Plants

*S*urface-disinfected *V. unguiculata* seedlings were transferred to 150 ml tubes with slopes of agarized Norris solution and 15 tubes were inoculated with 1 ml of sugarcane root suspension each. Negative controls (three plants) received 1 ml sterile saline solution and positive controls were inoculated with 1 ml of a suspension of strain BR3299 ($OD_{600} = 0.1$) (Radl et al., 2014). The plants were grown in a growth chamber at 25 °C with 12 h photoperiod under artificial light. Sterile water was added when necessary. Thirty days after inoculation, the occurrence of nodulation was evaluated. Nodules were collected and superficially disinfected by immersion in ethanol (70%) for 2 min, followed by treatment with undiluted commercial bleach for 3 min. After washing six times with sterile water, nodules were crushed on YMA plates, which were incubated at 28 °C for 7 days. Purity of bacterial isolates was checked by repeated streaking on fresh plates. Trap-plant isolates received names based on the number of the trap plant they were isolated

from, followed by a sequential number, as follows: P (# *V. unguiculata* plant - # isolate). Representative isolates were individually inoculated on *V. unguiculata* plants to check the nodulation capability, using the same methodology.

94.2.5 Isolation of Rhizobia from Sugarcane Tissues by Direct Plate Cultivation

Aliquots of 100 µl of 10^{-1} and 10^{-2}-fold diluted root extracts were dispersed on plates with modified YMA, containing as the carbon source mannitol (1 g/l) or a combination of the *Bradyrhizobium* spp. preferential carbon sources arabinose (0.5 g/l) and sodium gluconate (0.5 g/l) (Tong and Sadowsky, 1994). Incubation was performed at 28 °C for up to 21 days and slow-growing isolates were selected. Isolates were named as M or AG (mannitol or arabinose/sodium gluconate, respectively) followed by sequential numbers.

94.2.6 Genotypic Grouping of the Isolates by ERIC-PCR

ERIC-PCR was carried out with cell suspensions of the isolates under study as template in 20 µl volumes containing GoTaq® DNA Polymerase (Promega) and primers ERIC 1 (5′ATGTAAGCTCCTGGGGATTCAC 3′) and ERIC 2 (5′AAGTAAGTGACTGGGGTGAGCG 3′), basically as described previously (Woods et al., 1993). After PCR cycling, aliquots of 10 µl were loaded on 2% agarose TAE gels and electrophoresed for 4 h at 80 V. The molecular weight marker 1 kb plus ladder (Invitrogen) was used as reference. To determine genetic relatedness of isolates, a dendrogram was constructed based on the gel images that were analyzed using BioNumerics software version 6.6 (Applied Maths, Belgium), as described by Rademaker et al. (2000) with the DICE index, by the unweighted pair-group method of arithmetic averages (UPGMA). Optimization and tolerance levels were adjusted to 1.75.

94.2.7 PCR, Sequencing of 16S rRNA and *nifH* Genes, and Phylogenetic Analysis

PCR reactions were conducted using cell suspensions in sterile ultrapure water as template with GoTaq® DNA Polymerase (Promega, USA). 16S rRNA genes were amplified using the primers 27F and 1492R (Lane, 1991). The *nifH* gene was amplified with primers nifHF and nifHI (Laguerre et al., 2001). Reactions of isolates that did not produce amplicons using primers nifHF and nifHR were submitted to PCR with primers PolF and PolR (Poly et al., 2001). The PCR products were partially sequenced using primer 1492R for 16S rRNA and primer nifHI for the *nifH* gene using an ABI 3730xl DNA analyzer (Applied Biosystems, Germany). The 16S rRNA sequences (accession numbers KF113075-KF113106) and the *nifH* sequences (accession numbers KF113052-KF113074) have been deposited in the NCBI GenBank.

All sequences were submitted to blast analyses (Altschul et al., 1997), and for phylogenetic analyses, the sequences from reference strains and sequences obtained in the present study were aligned and analyzed using the ARB software (Ludwig et al., 2004).

94.2.8 Acetylene Reduction Assay (ARA)

For ARA, JMV medium (Reis et al., 2004) with some modifications was used, containing (in g/l) 5.0 mannitol, 0.6 K_2HPO_4, 1.8 KH_2PO_4, 0.2 $MgSO_4$, 0.1 NaCl, 0.02 $CaCl_2 \cdot 2H_2O$, 0.002 $Na_2MoO_4 \cdot 2H_2O$, 0.00235 $MnSO_4 \cdot H_2O$, 0.0028 H_3BO_3, 0.00008 $CuSO_4 \cdot 5H_2O$, 0.00024 $ZnSO_4 \cdot 7H_2O$, 0.0001 Biotin, 0.0002 pyridoxol-HCl, 0.0656 Fe-EDTA, 1.6 agar, pH 6.5. Three dosages of nitrogen source were tested: 0, 0.04, and 0.4 g/l yeast extract. Serum vials (20 ml) were filled with 10 ml semisolid media, inoculated with 20 µl cell suspensions in sterile water and closed with cotton plugs. After 5 days incubation at 30 °C, vials were sealed with rubber stoppers and 10% of the air space was replaced with acetylene gas. After 3 h incubation at 30 °C, 0.5 ml aliquots of the gas phase were analyzed by gas chromatography with flame ionization, using Perkin Elmer F11 equipment with a 50 cm Poropak N column at 70 °C. Nitrogenase activity was determined, after comparison to a standard curve, as the quantity (nmol) of ethylene produced per vial during the 3 h incubation period.

94.3 RESULTS

94.3.1 Cultivation-Independent Assessment of Diazotrophs in Sugarcane Cultivar RB867515

The characterization of the bacterial community colonizing sugarcane roots and leaf sheaths was first investigated using general primers for 16S rRNA-gene to obtain an overview about the entire communities in the greenhouse experiment. Four weeks after inoculation of the sugarcane plants (cultivar RB867515) with the 5-strain inoculum of Embrapa Agrobiologia, RNA was extracted from roots and leaf sheaths. The composition of bacteria in the samples is presented in Figure 94.1.

The predominance of alpha-proteobacteria is up to 70% especially in the inoculated roots, followed by beta- and delta-proteobacteria. Among the alpha-proteobacteria, sequences of *Acetobacteriaceae*, *Rhodospirillaceae,* and *Bradyrhizobiaceae* were identified. In the leaf sheaths also

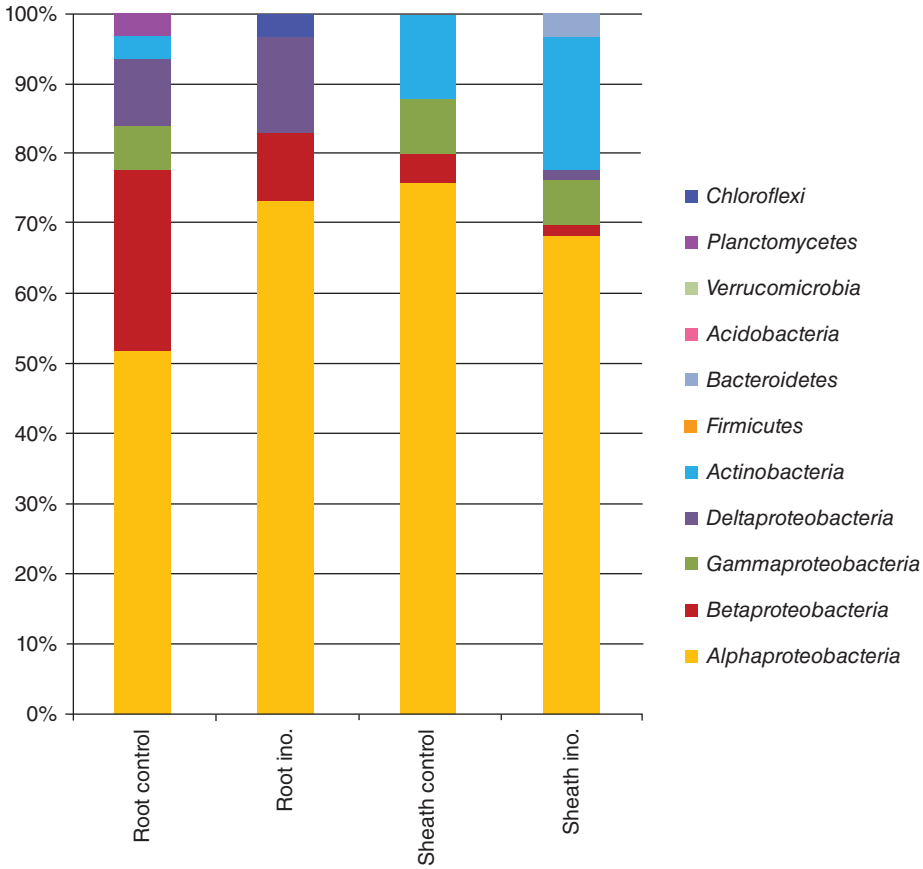

Figure 94.1 Bacterial composition (based on 16S rRNA cDNA) in root and leaf sheath samples after 4 weeks under greenhouse conditions.

alpha-proteobacteria dominated; after inoculation with the bacterial mixture, *Herbaspirillum* sp. *Gluconacetobacter* and *Bradyrhizobium* were found. Thus, there were indications that the inoculum may have been established, but obviously there were other bacteria, such as bradyrhizobia, regularly present, which may have come from the plant material.

In the field experiment (Fig. 94.2) performed at the field station of Embrapa-Agrobiologia in Seropédica, the effect of N-fertilization and inoculation with the 5-strain bacterial mix was investigated (Fig. 94.3).

At the harvest in March 2008, the difference between 16S rRNA DNA, representing the bacterial community, and cDNA, representing the active bacterial community, is only visible in some subpopulations. Within the alpha-proteobacteria, no sequences could be found that were related to *Gluconacetobacter diazotrophicus* or *Azospirllum amazonense*, which were part of the inoculum. Also, neither *Herbaspirillum seropedica* nor *Burkholderia tropica*-related 16S rRNA DNA-sequences could be identified, while other *Burkholderia*-, *Herbaspirillum*- and *Ralstonia*-related sequences were found (data not shown). Apparently, the strains from the inoculum were not part of the dominating community at the time of sampling (March, late summer).

Figure 94.2 Field trial at Embrapa Agrobiologia, Seropedica, 2008.

However, there were a number of clone sequences identified related to *Bradyrhizobium* spp. (Fig. 94.4) and other Rhizobiales (not shown).

16S cDNA and the *nifH*-transcripts in sugarcane roots and leaf sheaths of the same fields were investigated in March 2009. Reinoculation with the 5-strain inoculum was performed after the first cut in 2008. A high diversity of 16S cDNA and *nifH*-transcripts could

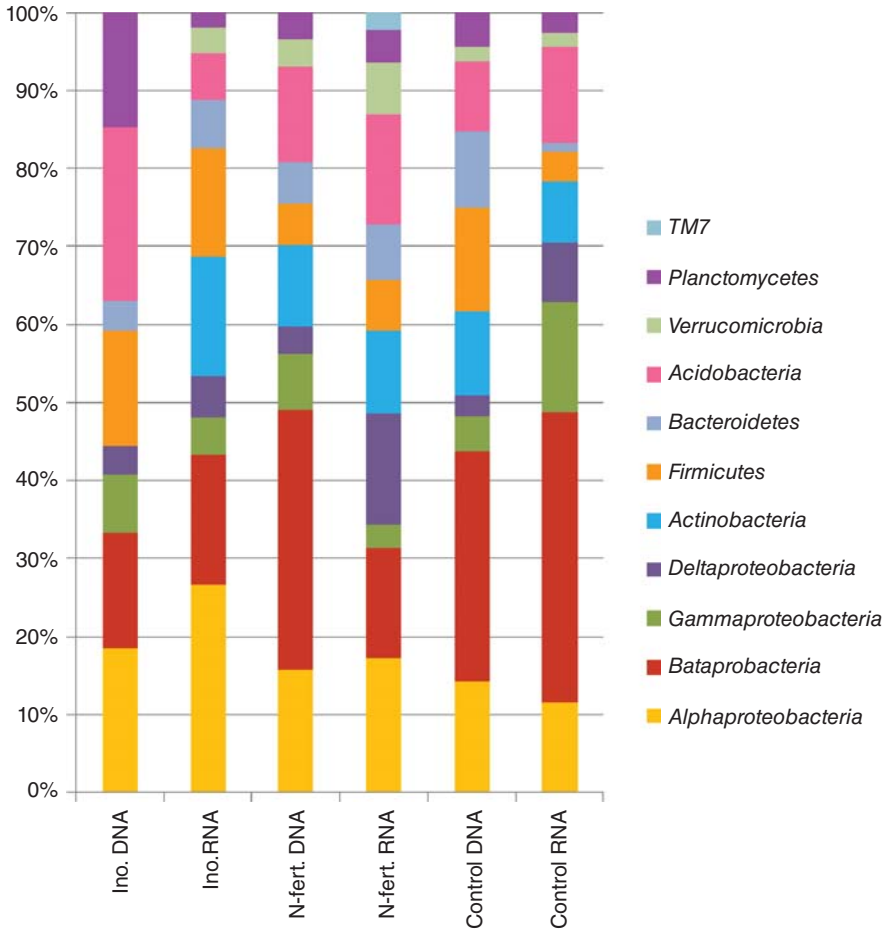

Figure 94.3 Bacterial composition (16S rRNA sequence diversity from RNA and DNA) from root samples of the field trial 2008.

be retrieved from these sugarcane plants (Fischer et al., 2012). *Gluconacetobacter diazotrophicus nifH*-cDNA, as well as 16S cDNA-sequences identical to the inoculated strain were found in ample amount in leaf sheaths of inoculated sugarcane plants (Fischer et al., 2012). However, 16S cDNA and *nifH*-cDNA of the other inoculant strains could not be found. A detailed sequence analysis of the *nifH*-sequences of each of the inoculation strain revealed that *Azospirillum amazonense* CBAmC as well as *A. amazonense* DSM2787 harbor a *Bradyrhizobium*-like *nifH*-sequence. However, this sequence could also not be found within the *nifH* cDNA-sequences retrieved from the sugarcane plants. However, 16S cDNA- and *nifH* cDNA sequences of a new type of bacterial species within the *Ideonella–Azohydromonas–Herbasprillum* cluster was found very frequently mostly in leaf sheaths (not shown). Besides other not yet cultured bacteria from the *Burkholderiaceae*, many 16S cDNA and *nifH* cDNA-sequences from *Bradyrhizobium* were found in roots (Fig. 94.5). *Rhizobium* spp. sequences were retrieved mostly from leaf sheaths (Fischer et al., 2012).

94.3.2 Isolation and Characterization of *Bradyrhizobium* sp. from Sugarcane Cultivar RB867515 by Direct Plate Cultivation and by Using *V. unguiculata* Trap Plants

On the basis of the results obtained with culture-independent approaches, we conducted experiments aiming to isolate bacteria from the genus *Bradyrhizobium* from sugarcane tissues, by inoculating extracts of surface-disinfected sugarcane roots directly on plates or on *V. unguiculata* (cowpea) trap plants. After the disinfection procedure of sugarcane roots, no bacteria grew when water of the final washing step was spread on YMA plates or when disinfected and washed root fragments were placed on YMA plates. This suggested that the procedure was effective in removing the root surface-associated microbial community.

Thirty days after individually inoculating 15 *in vitro* grown *V. unguiculata* plants with 1 ml of the extract obtained by macerating the disinfected roots, 13 of these plants presented nodules from which bacteria could be isolated.

Figure 94.4 Phylogenetic tree (neigbor-joining) of 16S rRNA gene of *Bradyrhizobium* spp. Sequences were obtained from the field trial 2008 (purple), from plate isolation approach (green), and trap plant approach (blue).

Three un-inoculated control plants did not present nodules, whereas the positive control nodulated abundantly. From the nodules formed on the 13 plants inoculated with sugarcane root extract, a total of 112 bacterial isolates was obtained (Rouws et al., 2014). These isolates received names as follows: P followed by [number of *V. unguiculata* plant]-[isolate number]. The large majority (109 isolates) grew slowly and alkalinized the culture medium, characteristics typical of the genus *Bradyrhizobium* (Kuykendall, 2005). Three isolates (P5-2, P5-21 and P5-25) were fast-growing and showed a neutral/acidic reaction on YMA. These data indicated that *V. unguiculata* nodule-inducing bacteria occur as endophytes in roots of sugarcane cv. RB 867515.

ERIC-PCR fingerprinting was applied to identify genotypic redundancy among the 112 isolates. When all isolates/strains were grouped with a low level of correlation (28% similarity) this analysis revealed 28 distinct ERIC profiles. Some identical fingerprint profiles were shared by up to 36 isolates, showing that some genotypes were redundant in the collection. Isolates sharing identical ERIC-PCR profiles were obtained from nodules of the same plant, but also from nodules of independent *V. unguiculata* plants inoculated with the same aliquot of sugarcane root extract. Some ERIC-PCR profiles (13) represented fewer isolates and several profiles represented unique isolates in the collection. The relative abundance of certain ERIC-PCR profiles may reflect the abundance of the respective bacterial genotype in the sugarcane roots, differences in colonization efficiency of the *V. unguiculata* trap plants by different bacterial genotypes, or differences in bacterial growth rate.

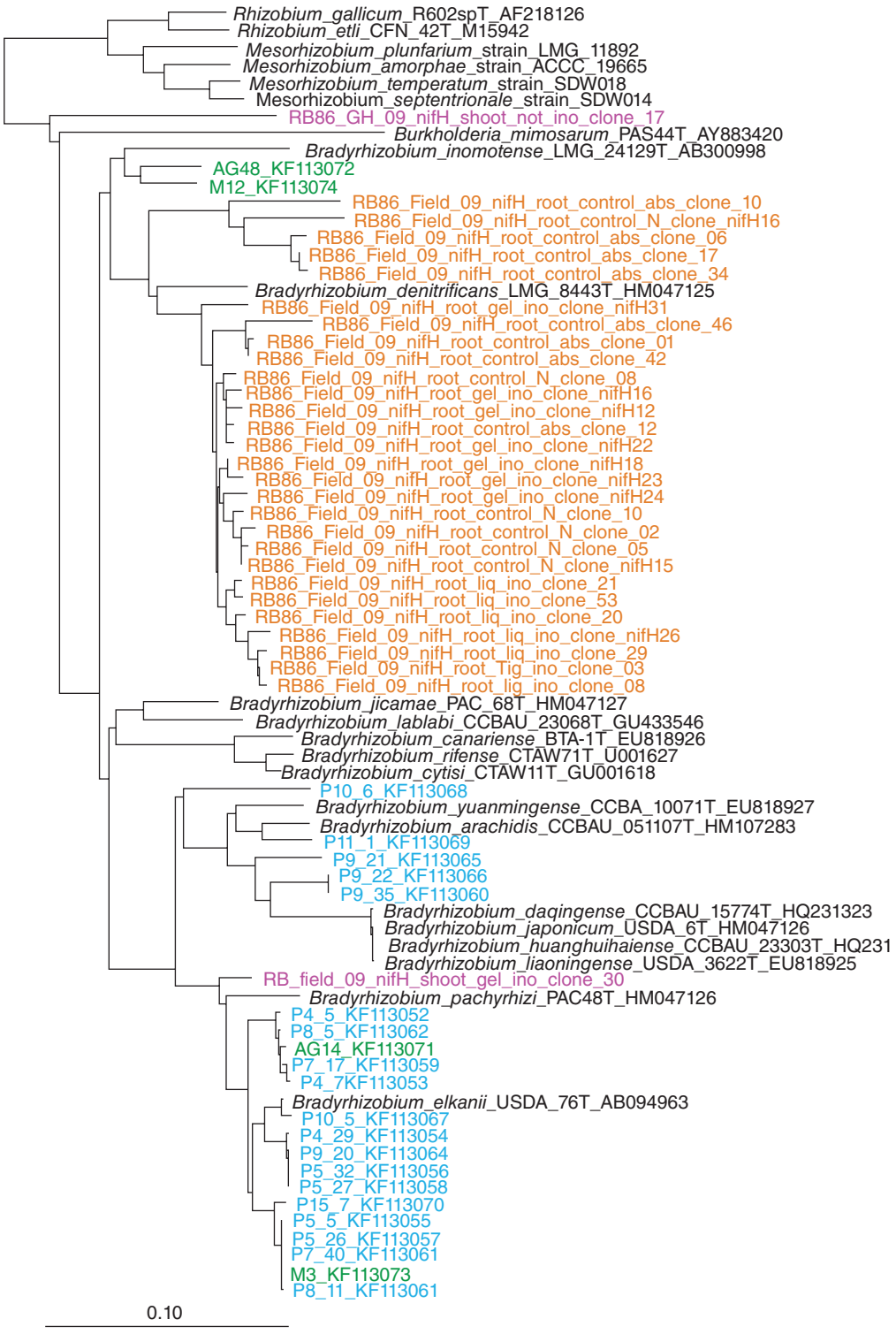

Figure 94.5 Phylogenetic tree (neigbor-joining) of *nifH* of *Bradyrhizobium* spp. Sequences were obtained from the greenhouse trial 2009 (pink), from the field trial 2009 (orange, see Fischer et al. (2012)), from plate isolation approach (green), and trap plant approach (blue).

Based on the ERIC-PCR profiles, 23 representative isolates were selected for more detailed analyses.

In addition to these trap plant isolates, the direct plate cultivation strategy permitted the selection of eight slow-growing and one fast-growing *nifH*-containing isolates (determined by PCR analysis). Sequence analyses of the 16S rRNA gene of these 32 isolates confirmed that 28 belong to the genus *Bradyrhizobium*, 1 isolate (AG46) was most closely related to the genus *Methylobacterium*, 1 isolate to the genus *Herbaspirillum* (AG47), and the 2 fast-growing isolates (M30 and P5-2) grouped together with species from the genus *Rhizobium* (Fig. 94.4).

When individually inoculated on *in vitro* grown *V. unguiculata* plants, all but one (the fast-growing isolate P5-2) trap plant isolates were able to nodulate. However, among the direct plate isolates only M3 and AG14 were able to induce nodules, whereas the other *Bradyrhizobium* spp. isolates (M12, M21, AG41 and AG48) were not. This observation demonstrated that the culture strategy had a significant influence on the isolation of specific groups of bacteria. It also showed that sugarcane plants harbor besides nodulating *Bradyrhizobium* spp. also Bradyrhzobia that are unable to nodulate a promiscuous legume such as *V. unguiculata*.

For 21 of these selected isolates, the partial *nifH* gene was sequenced and the sequences were aligned with *nifH* genes from reference strains and with the *nifH* cDNA sequences described in the present study (Fig. 94.5). These analyses showed that de *nifH* gene of most isolates, including the nodule-inducing direct plate isolates M3 and AG14, clustered together in a branch with the *B. elkanii* strain USDA 76^T. It was interesting to note that the *nifH* gene of the other two *Bradyrhizobium* spp. direct plate isolates M12 and AG48 were quite dissimilar from this cluster and were more similar to some *nifH* cDNAs obtained in this study. This emphasized that the culture strategy may greatly influence the diversity of bacteria recovered from an environmental sample.

Twelve representative isolates were tested for *in vitro* nitrogenase activity by ARA. The majority (M3, AG14, P5-2, P8-11, P10-5, P11-1 and P15-7) did not present any detectable activity (data not shown). However, four isolates presented significant nitrogenase activity in a semisolid medium containing 0.4 g/l yeast extract, as determined by the quantity of ethylene produced: M12 (7 nmol), AG48 (51.7 nmol), P9-20 (17.7 nmol), and P9-21 (3.5 nmol). Therefore, some sugarcane endophytic *Bradyrhizobium* spp. could fix nitrogen without being associated to a nodule environment, a prerequisite for sugarcane-associated BNF.

94.4 DISCUSSION

The important contribution of microbes to overall plant performance was revived in recent years when the term "plant microbiome" became popular (Turner et al., 2013). Metagenomic approaches enabled the analysis of plant associated microbes, including the endophytic microbial community, with high throughput genome sequencing approaches.

In energy crops, the awareness of the importance of bacterial impacts on plant nutrition and health goes back much longer. Fostered by the successful discoveries by the group of Johanna Doebereiner of several new diazotrophic bacterial genera and species several decades ago, energy plants such as sugarcane were also investigated in detail (Döbereiner, 1961; Döbereiner, 1995; see Chapter 88). In *Pennisetum purpureum,* another promising energy plant for the tropics, thorough estimates of ^{15}N natural abundance measurements demonstrated similar high rates of BNF in some cultivars as was found in sugarcane (Urquiaga et al., 2012; Morais et al., 2012). Using cultivation-based approaches, a diversity of diazotrophic bacteria were found in several cultivars of field-grown *Pennisetum* (Videira et al., 2012). Based on enrichment and isolation studies on nitrogen-free semisolid media – such as NfB, JNfB, LGI – *Enterobacter cloacae* and other *Enterobacter* spp. were isolated, which were shown to harbor *nifH*-genes. Besides *Gluconacetobacter diazotrophicus*, diazotrophic *G. sacchari* were also characterized for the first time, which apparently had acquired their *nifH*-genes from *Enterobacter* spp. colonizing *P. purpureum.* When the diversity of diazotrophs was studied with a cultivation-independent approach by cloning the 16S rDNA and *nifH*-gene from roots and stems of *P. purpureum*, a much higher diversity of diazotrophic community, became apparent (Videira et al., 2013). After the experience with sugarcane and the demonstration of a considerable occurrence of *Bradyrhizobium* and *Rhizobium* sequences, it was not a surprise to find *nifH*-transcripts from these bacterial genera also in *Pennisetum* cultivars (Videira et al., 2013). Therefore, similar research efforts should also be brought forward in *Pennisetum* to isolate *Bradyrhizobium* spp. using the legume trap plant approach and other more direct plate isolation techniques, as were applied in sugarcane (Rouws et al., 2014). It is quite obvious that the successful approach of enrichment and isolation of diazotrophic bacteria using nitrogen-free semisolid media leads to the isolation of only a subfraction of the whole community of diazotrophic bacteria from non-leguminous plants.

The occurrence of *Rhizobium* and *Bradyrhizobium* sp. in non-leguminous plants had attracted already quite intense attention, especially in cropping systems, where a monocot crop (such as rice or wheat) and legumes (clover or soybean) are used in alternation for hundreds of years, such as in the Nile valley and delta in Egypt (Yanni et al., 2001; see Chapter 111). A diversity of *Rhizobium leguminosarum*

bv.trifolii isolates could be obtained from rice roots, which colonize rice plants endophytically and exert a plant-growth promoting effect. *Rhizobium leguminosarum* was also shown to colonize roots of wheat and other non-leguminous plants (Schloter et al., 1997). While stimulation of growth of rice by selected *R. leguminosarum* bv. trifolii strains could be demonstrated repeatedly (Yanni et al., 2001; see Chapter 111), evidence for *in planta* nitrogen fixation could not be found. Photosynthetic bradyrhizobia have also been isolated from the African wild rice *Oryza breviculata* (Chaintreuil et al., 2000). A recent study reported on the isolation of an endophytic *Bradyrhizobium* sp. isolate (strain AT1) from sweet potato and ARA analyses and ^{15}N-isotope dilution studies suggested that this strain might increase plant growth through BNF (Terakado-Tonooka et al., 2013).

As demonstrated in our study, a high diversity of *Bradyrhizobium* and *Rhizobium* bacteria was found active as nitrogen-fixing bacteria in sugarcane, because a large number of transcripts of the *nifH*- and also 16S rRNA genes were found in roots and stems of field grown sugarcane variety RB867515. This constitutes evidence for an important role of these bacteria under natural conditions. Therefore, attempts are now under way to inoculate axenically grown sugarcane plantlets with the *Bradyrhizobium* spp. isolates originating from the same sugarcane cultivar and to demonstrate not only efficient endophytic colonization but also *in situ* active nitrogen fixation. Detailed laboratory studies of these bacteria may find specific adaptations to the metabolic scenario of sugarcane to finally establish an efficiently nitrogen-fixing symbiotic system. A final proof of *in planta* nitrogen fixation would be to demonstrate fixation of $^{15}N_2$-gas by sugarcane plants inoculated with *Bradyrhizobium* spp. isolates originating from sugarcane.

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Chapter 95

How Fertilization Affects the Selection of Plant Growth Promoting Rhizobacteria by Host Plants

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95.1 INTRODUCTION

Currently, 40% of the world's population is fed by the increased crop productivity that results from the use of fertilizers (UNEP, 2007). However, fertilizer production is dependent on limited fossil fuels, the use of which is harmful to vital natural resources. This dependence creates a completely unsustainable situation that will prevent humanity from maintaining its current food production system: it is estimated that food production worldwide will either change or collapse within a few years. Obviously, this issue requires the attention of the scientific community.

Fertilizers supply plants with important nutrients, allowing growth and productivity that is much greater than those obtained with unfertilized plants. Chemical fertilizers were first used in the mid-nineteenth century in Europe, when low levels of phosphates in the soil endangered continuous food production in industrialized countries (Dawson and Hilton, 2011; Neset and Cordell, 2011). Traditional organic fertilization did not recycle enough phosphate to replace what was removed during increasingly large harvests in lands that had been cultivated for centuries. The discovery of rock phosphate as an amendment to crops in 1840 allowed the continuity of the agricultural system, avoiding a food production crisis for 1.5 billion humans on earth (Dawson and Hilton, 2011). However, by the start of the twentieth century, the lack of biologically available nitrogen for crops presented a new challenge for scientists and farmers. The Haber–Bosch process, developed in 1909, enabled ammonia to be synthesized from atmospheric nitrogen and to be used in crop production (as well as in explosives and ammunition production, as World War I was about to occur). In subsequent years, man-made reactive nitrogen greatly increased food production, and the inventors of the ammonia synthesis process received two Nobel prizes, one in 1918 and another in 1931. After the food shortages caused by World War II, there was intense investment in crop production policies, especially in enhancing plant responsiveness to fertilizers, greatly increasing the productivity and profitability of farming systems (Dawson and Hilton, 2011). This new technological package (heavily dependent on fertilizers, pesticides, herbicides, and fertilizer-responsive high-yield plant varieties) was adopted by several developing countries, giving rise to the Green Revolution (Evenson and Gollin, 2003). These developments greatly improved food security and prevented starvation of millions of people, but they have come with a steep cost to nature (see Chapters 5, 109).

The excessive use of pesticides has damaged many ecosystems and caused health problems for human workers, and the high-yield varieties of rice, wheat, corn, and soybeans developed in Europe and the United States have resulted in decreased use of native varieties and crops. Furthermore, local farming has become overshadowed by industrialized farming. Agriculture has grown completely dependent on the use of chemical NPK fertilizers to ensure food production at the required levels. However, the

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production of fertilizers is dependent on non-renewable resources. Despite manifold improvements, the Haber–Bosch process still used today consumes natural gas to break down the strong triple bond of atmospheric dinitrogen and to supply free H to form two molecules of NH_3 out of N_2 and CH_4, consuming about 1.1% of the entire world's energy production (Dawson and Hilton, 2011; FAO, 2011). There is no other feasible source of reactive nitrogen at an industrial scale: it cannot be harvested, extracted, or mined. Its only source is chemical synthesis, and the only economically viable fuel for its production is natural gas. Reactive nitrogen can also be produced through water hydrolysis, but such an approach is not yet cost-effective.

Nevertheless, nitrogen is not the only finite source that humanity utilizes to produce food: the reserves of rock phosphate, the main source of P for fertilizers, are also limited and non-renewable. The largest reserves of rock phosphate are found in only five countries, and 77–85% of the phosphate supply is found only in Morocco (which holds a significant part of this reserve in a conflict zone). Approximately 90% of all phosphate mined on earth is used for fertilizer production, but only approximately 20% of the phosphate removed from the reserves ever reaches the consumers' tables, illustrating the remarkable inefficiency of the entire production process. The peak of rock phosphate production might be reached as soon as 2033 – from this point on, prices should increase while supply decreases (Dawson and Hilton, 2011).

Potassium is the least troublesome of the NPK nutrients. It is also mined from underground, but salty lakes (Römheld and Kirkby, 2010) or even ocean waters could be used as a secondary and renewable source of potassium because it is found at naturally high concentrations in salty bodies of water (400 mg/l of K against 0.06 mg/l of P) (Dawson and Hilton, 2011). Additionally, high concentrations of potassium in soils and water are not harmful to human health nor do they threaten wildlife (Ramasamy et al., 2005); in addition, they usually do not create detectable environmental damage.

Fertilizers applied to crops can also run off because significant portions of the introduced nutrients are not taken up by crops and escape to the environment, causing damage. Depending on the soil characteristics, 0.4–90% of the applied fertilizers might not be taken up by crops, with losses of 50% being very common (Adesemoye and Kloepper, 2009; Simpson et al., 2011). This means that millions of tons of expensively processed nutrients are washed away by rain or volatized to the atmosphere, causing nutrient imbalances around the world. Biologically active nitrogen and phosphate can reach water bodies, creating disturbances in nutrient cycling and availability and causing eutrophication in rivers, lakes, and coastal waters. An excess of P causes algal blooms of cyanobacteria (which are often toxic) because P is the major nutrient that limits their development. In P-rich conditions, a few cyanobacteria outcompete other phytoplankton species and drastically change the biological component of water bodies. Eutrophication has a negative impact on public health, the economy, and the environment, including water hypoxia (consumption of dissolved oxygen by bacterial decomposition, which leads to fish death by suffocation), changes in trophic webs, biodiversity loss, blocking of sunlight from the water column, toxic algae blooms, changes in water quality for human consumption, and other such effects (Carpenter et al., 1998; Lau and Lane, 2002). Many coastal waters are in severe danger, as this type of damage is difficult to control because it is non-punctual (unlike sewer water from cities, which has a punctual geographical origin) and is difficult to counter because it affects entire environments.

While P causes severe damage to water bodies, reactive nitrogen can be volatized to the atmosphere by microbial nitrification and denitrification, causing damage on a global scale. These volatized gases (especially N_2O) might return to the soil in regions far away from farming centers, creating nutrient unbalances, altered growth rates, and biodiversity changes (UNEP, 2007). Approximately 75% of all anthropically generated N_2O comes from agriculture. N_2O is a very dangerous greenhouse gas because it is very stable (it survives in the atmosphere for approx. 100 years) and very potent (1 g of N_2O has the global warming potential of 300 g of CO_2) (UNEP, 2007). Another problem that agriculture faces is the reduction in farming lands, meaning there is the need for even greater productivity. The world's best areas for high crop productivity due to good soil conditions were historically adjacent to cities, but such farms are quickly being replaced by urban expansion. Even if the best agricultural practices are used on the most fertile lands, the financial gain from farming cannot compete with the financial gain from turning these lands into high-profile residential areas (Singh et al., 2011).

The production and release of artificially produced reactive nitrogen compared to the amount of nitrogen fixed naturally by diazotrophs gives us a picture of the scale of the problems generated by these processes. The continual increase in the use of fertilizers worldwide has resulted in a release of reactive nitrogen that is now equal to the amount of naturally fixed nitrogen worldwide, seriously threatening the natural cycling of this nutrient. Approximately 100 million tons of both man-made and natural reactive nitrogen are produced every year, effectively doubling the amount of nitrogen available to the biosphere. This is one of the most disrupted natural cycles man has interfered with, and this disruption will seriously compromise precious water resources sooner rather than later if left unchecked (FAO, 2011; UNEP, 2007).

Thus, humanity faces a truly unsustainable situation: it is completely dependent on an environmentally dangerous (with respect to both use and production), limited natural resource, the supply of which is expected to decline sharply in less than 50 years. This situation cannot continue for an indefinite time. Thankfully, there are many alternatives to

this traditional system, including improved techniques and materials for fertilizer use and production. One very promising technology is the utilization of plant growth-promoting rhizobacteria (PGPR), which can easily reduce up to 50% of the use of fertilizers on a crop without any loss in productivity (Baldani et al., 1986; Alves et al., 2003; Hayat et al., 2010; Good and Beatty, 2011; Miransari, 2011; see Chapters 88, 90, 92).

95.2 THE EFFECT OF FERTILIZERS ON THE USE OF PGPR

95.2.1 Fertilizers Reduce Nitrogen Fixation by PGPR

The use of fertilizers influences the relationship between PGPR and their host plants. Nitrogen fixation by diazotrophs may be reduced or even halted by the use of nitrogen fertilizers in soils because the presence of reactive nitrogen in the soil acts as a repressor for the nitrogen fixation mechanism. Because the whole cascade is energetically expensive for the bacteria, they will not activate their nitrogen fixation mechanism if it is much easier to simply take the reactive nitrogen from the soil. It has been widely reported that nitrogen fertilization reduces the number and activity of root nodules in legumes and affects the diversity of *nifH* genes in microbial populations (Kolb and Martin, 1988; Muthukumarasamy et al., 2007; Prakamhang et al., 2009). This issue has already been extensively discussed and is not the focus of this review.

95.2.2 Fertilizers Interfere with Plant Selection of PGPR with Respect to Two Traits Simultaneously: Phosphate Solubilization and Auxin Production

Previous work from our group (Costa et al., 2012) demonstrated an interesting correlation between phosphate solubilization and the indolic compound production abilities of bacteria isolated from different NPK fertilization conditions. Root-associated diazotrophs isolated from rice plants under unfertilized conditions have shown higher phosphate solubilization activity and lower indolic compound production, while root-associated bacteria isolated from rice plants under light fertilization conditions (50% fertilizer dose) have shown higher indolic compound production and lower phosphate solubilization activity. In addition, the indolic compound production of rhizospheric bacteria isolated from rice plants grown under unfertilized conditions was higher than the indolic compound production displayed by root-associated bacteria, and the phosphate solubilization ability was higher in rhizospheric bacteria isolated from rice plants under light fertilization conditions compared to the root-associated bacteria. Plants were effectively hosting bacteria based on their plant growth-promoting traits (PGP) characteristics: nutrient solubilization over hormone production in poor nutrient conditions and hormone production over nutrient solubilization in moderate nutrient conditions. Rhizospheric and root-associated bacterial populations isolated from full nutrient conditions (100% fertilizer dose) were very similar with respect to their phosphate solubilization activities and indolic compound production, suggesting that at such fertilization levels, these two PGP traits were not important factors for plant-driven bacterial selection. We also found an inverse correlation between these two PGP traits: good phosphate solubilizers were not good indolic compound producers, and good indolic compound producers were not good phosphate solubilizers. Still, the isolates could present an intermediary phenotype or poor expression of both PGP traits. There appears to be no naturally occurring good phosphate solubilizers that are also good indolic compound producers. These findings have a series of implications.

First, plants select bacteria according to their specific PGP traits in response to the specific nutrient availability. It is not of benefit to the plant to host better hormone producers (found in the rhizosphere) in a situation where nutrients are more limiting. Once nutrients were more readily available, the plant left the best phosphate solubilizers in the rhizosphere and kept the best hormone producers inside the tissues. This result could mean that nutrient solubilizers are better PGPR in nutrient-poor conditions and that hormone producers are better PGPR in nutrient-moderate conditions. When we tested some selected strains in a field experiment, the good phosphate solubilizers (originally isolated from unfertilized conditions) could only enhance plant growth under unfertilized conditions, while the good growth hormone producers (originally isolated from light fertilization conditions) only improved plant growth under light fertilization conditions (Costa et al., 2012). Under heavy fertilization conditions, these two PGP traits were not driving factors for plant growth. It is interesting to note that the isolates promoted growth only in the environments they were isolated from and that PGPR prospection usually takes bacteria from conventional crop fields (under 100% fertilizer) and tests them for optimal use at the 50% fertilizer level. These findings help us to better select PGPR candidates according to the target field conditions as well as help us to direct the PGPR bioprospection according to the PGP trait of interest.

Results similar to those described above have already been reported. Poonguzhali et al. (2006) studied the effects of organic and chemical fertilization on rhizospheric and endophytic isolates of Chinese cabbage. While the focus of their work was on the principal component analysis (PCA) grouping of taxonomic and substrate utilization characteristics of

the microbiota, a factorial analysis considering the interaction effect between the niche and the fertilization condition was missing. Using the data available from this paper, we performed a two-way ANOVA analysis of the indolic acetic acid (IAA) production ability of the isolates in relation to niche and fertilization interaction effects. There was a significant interaction between these factors ($p = 0.04$), indicating that the IAA production ability was higher for the endophytic isolates than for the rhizospheric isolates when they were subjected to chemically fertilized conditions, but in the unfertilized condition, the rhizospheric isolates were the best indolic compound producers. Dias et al. (2008) also studied the IAA production and phosphate solubilization abilities of endophytic bacteria isolated from strawberry plants. He reported that the best indolic compound producers were not the best phosphate solubilizers. These studies agreed with our proposed model for IAA production. It has already been reported that the application of phosphate fertilizer at many different levels may increase the general bacterial biomass, including phosphate solubilizers (Hu et al., 2009; Chu et al., 2007; Gu et al., 2009). In addition, phosphate fertilization may reduce the phosphate solubilization activity and potential of the microorganisms (Hu et al., 2009) because they no longer need to expend so much energy to solubilize a more readily available resource.

95.2.3 Effects of Fertilization on Siderophore Production

Although the effects of different nitrogen fertilizers on rhizosphere pH is well documented (Kurek and Jaroszuk-Ściseł, 2003), there is little published work regarding the effects of fertilization on siderophore production, even if pH affects iron bioavailability directly. A previous work (Costa et al., 2012) showed that rhizospheric bacteria isolated from heavy fertilization conditions presented higher siderophore solubilization indexes (a ratio of halo and colony size on a plate trial) than endophytic bacteria isolated from the same condition, while both rhizospheric and endophytic bacteria isolated from light fertilization and unfertilized conditions presented similar siderophore solubilization indices. Another report showed that there were no differences in siderophore production due to the addition of straw or manure to crop soil when compared to an untreated control (Rodgers-Gray and Shaw, 2001). A previous work also suggested that there was a slight correlation between siderophore production and phosphate solubilization (Costa et al., 2012) that needed further confirmation. Perhaps the increase in siderophore production due to fertilization is linked to the higher bacterial biomass and diversity caused by the use of fertilizers, as siderophore production is related to bacterial competition and exclusion due to iron nutrition limitation (Weyens et al., 2010).

95.2.4 Fertilizers Reduce the Activity of ACC-Deaminase by Bacterial Isolates

Some bacteria may produce the enzyme 1-aminocylopropane-1-carboxylate (ACC)-deaminase, which hydrolyzes ACC to α-ketobutyrate and ammonia. ACC is the immediate precursor of ethylene, an important plant hormone that regulates growth according to its concentration and the plant's physiological condition (Arshad and Frankenberger, 2002). Because high levels of ethylene are expected to inhibit root development, bacteria that prevent its synthesis by hydrolyzing the immediate precursor ACC might help plant root development and growth, as has been shown in several studies (Long et al., 2008; Babalola, 2010; Miransari, 2011; see Chapter 71). Because ethylene production is sensitive to the nutrient status around the roots (Abeles et al., 1992) and increases under nutrient-deficient stress conditions (Glick et al., 1998; Jalili et al., 2009), one might expect that its production relates closely to fertilizer application, ultimately leading to shifts in the rhizobacteria community structure or function.

There is evidence suggesting that the use of fertilizers reduces the effectiveness of ACC-deaminase activity. Shaharoona et al. (2006) tested three strains belonging to the *Pseudomonas* genus that were selectively isolated from the maize rhizosphere for ACC production in field assays under different fertilization conditions. All treatments received P and K at 100 and 50 kg/ha, respectively, but N was added at 175 kg/ha only for some treatments. While two strains were able to induce plant growth even in the presence of the N fertilizer, their effectiveness was greater in the absence of the N fertilizer compared to the control. Furthermore, the third isolate tested promoted plant growth only in the absence of the N fertilizer. In their subsequent work (Shaharoona et al., 2008), two isolates of *Pseudomonas fluorescens* that presented ACC-deaminase activity were tested in greenhouse and field trials. In greenhouse trials, both isolates were able to increase plant root weight and biomass in the presence of 0, 25, 50, 75, and 100% NPK fertilizer of 120-100-60 kg/ha, respectively, but the efficiency of the inoculation clearly decreased with increasing fertilizer levels. This pattern was repeated in the field trials, as several yield measurements were improved by both isolates under different fertilization conditions, but their growth efficiency compared to the non-inoculated control was greater under lower fertilization conditions. There were significant negative correlations between the inoculation efficiency and the increasing fertilizer application for various yield parameters in both greenhouse and field trials. In Zabihi et al. (2010), four *Pseudomonas* strains presenting ACC-deaminase activity were tested on wheat plants in greenhouse and field trials under different P fertilization conditions of 0, 25, and 50 kg/ha. While the 25 kg/ha dose of P fertilizer generally

presented the best productivity with respect to yield, in some cases, the highest PGPR activity was achieved at zero P fertilization and with decreasing efficiency as the fertilization levels increased.

These reports suggest that ACC-deaminase becomes less important as the plant liberates less ethylene in response to stressful nutritional conditions. In fully fertilized conditions, less ethylene is produced, and ACC-deaminase no longer functions to elongate root length. This PGP trait activity ceases to be a very important trait of the rhizosphere community under such conditions. It is tempting to speculate that the number, activity, and niche occupation of ACC-deaminasing bacteria under different fertilization conditions might change.

95.2.5 Effects of Fertilization on Bacterial Motility

There is no information about the effects of fertilizer on bacterial motility, even though motility and bacterial dispersion are important factors for successful root colonization (Babalola, 2010; Turnbull et al., 2001b; Kristin and Miranda, 2013). Bacterial mobility is also related to nutrient availability, as nutrient limitation increases motility, and high nutrient concentrations reduce swimming speed and the selective advantages for higher motility (Mitchell and Kogure, 2006; Matz and Jürgens, 2003). A study by Turnbull et al. (2001a) showed that mobile *Pseudomonas* strains attached to roots more successfully than non-mobile strains in low-nutrient media, while high-nutrient media reduced general attachment by mobile and non-mobile strains to an equal extent. Thus, less mobile bacteria could survive in crop soils due to fertilization-induced increased nutrient availability, which ultimately might hinder plant–bacteria interactions. The exact impact of this possible interaction should be looked into, as nutrient excesses might generate "lazy" bacteria that are less suitable for use as PGPR.

95.2.6 Effects of Fertilization on Antagonism

Antagonism effects vary greatly because each single plant–antagonist–pathogen relationship is different. Several biotic and abiotic characteristics that influence rhizosphere composition might affect antagonistic relationships as well. For example, organic agriculture and the use of organic composts contribute to the improvement of soil quality, increase microbial richness and evenness, promote a decrease in pathogenic damage, and prevent diseases in the soil (Chaparro et al., 2012; Wu et al., 2008; Bulluck et al., 2002). However, the type of chemical or organic fertilizer as well as the type of compost used might greatly influence the effectiveness of antagonism (Kurek and Jaroszuk-Ściseł, 2003; Wu et al., 2008; Hoitink and Fahy, 1986). The enhancement of antagonism is more likely to occur with the use of organic fertilizers than chemical fertilizers, but this enhancement is still subject to many potentially disruptive biotic and abiotic factors.

95.2.7 Fertilizers Affect the Bulk Soil, Rhizospheric, and Endophytic Bacterial Populations in Different Ways

It has been established that plants select certain types of bacteria to execute specific functions (Hartmann et al., 2008). One can see this by looking at shifts in the associated microbiome following environmental changes or comparing the bacteria found in the different niches of the rhizobiome: endophytic and rhizoplane bacteria, rhizospheric bacteria, and bulk soil bacteria. These groups of bacteria represent different communities that are subjected to different selective pressures and challenges. As the physical distance from the roots increases, the total bacterial population decreases in number but increases in diversity. Bulk soil works as a "storage area" of very different bacteria, and a subset of the diversity found in bulk soil will be selected by plants to join the rhizosphere, creating the "biased rhizosphere." In the same manner, endophytic bacteria are a subset of the more diverse rhizospheric bacteria. Plants are expected to greatly control which bacteria will be found in which niche (Hartmann et al., 2008).

There are many reports that show differences between the taxa or function of endophytic and rhizospheric bacteria. Seghers et al. (2004) used a denaturing gradient gel electrophoresis (DGGE) approach with maize samples to show that type I methanotrophs were present in both endophytic and rhizospheric communities, while type II methanotrophs were present only in the rhizospheric community. In addition, the methanotroph diversity was higher after organic fertilization compared to chemical fertilization. Wang et al. (2009) detected significant differences between root-associated and rhizopheric ammonia-oxidant bacteria from rice under different N fertilization conditions. Most of the root-associated and rhizospheric samples clustered differently in a PCA when Terminal-restriction fragment length polymorphism (T-RFLP) patterns were compared. These authors observed a trend wherein the relative abundance of *Nitrosomonas* increased relative to *Nitrosospira* with the increase of N fertilization right after the application of fertilizers. Pariona-Llanos et al. (2010) showed that organic fertilization resulted in greater endophytic bacterial numbers than chemical or no-fertilization conditions in sugarcane. Two enzymes related to active penetration of plant cells were also screened, and it was found that pectinase activity was more present after chemical fertilization, while endoglucanase activity was more present after organic fertilization. Unfertilized conditions had lower occurrence of both traits.

Ferrara et al. (2011) studied endophytic and rhizospheric enterobacteria of the *Enterobacter* and *Klebsiella* genera isolated from sugarcane in commercial fields and compared some PGP traits. They found that amino acid release was greater in endophytic isolates while antagonism and nitrogen fixation were greater in rhizospheric isolates, but IAA production was similar in both niches. Many PGP traits can be differentially selected by plants under different conditions; thus, there are no "typical" endophytic or rhizospheric bacteria (besides the symbiotic legume-rhizobium), but instead, plants select bacteria according to their current needs.

95.3 CONCLUSIONS

As we can see, fertilization works differently on PGPR from different niches and on several different traits of the community. Community genera composition, bacterial concentration, enzyme production, antagonism, nitrogen fixation, nutrient solubilization, and genetic and functional diversity of rhizospheric and endophytic bacteria were all affected differently by fertilization. Fertilization affects many important PGP traits, community structure, and the efficiency of PGPR inoculation. Simple experiments with double-cross comparisons of endophytic and rhizospheric communities of fertilized and unfertilized conditions may still reveal many of the preferred PGP traits of the plants. The discovery of the selected attributes may greatly increase inoculation efficiency because researchers might be able to inoculate crops with the "more favored" PGPR under field conditions.

Endophytic and rhizospheric bacteria under fertilized and unfertilized conditions can be statistically compared in many ways. For continuous PGP traits, a two-way factorial ANOVA with a simple main effect analysis as a post-hoc test can reveal interesting interaction effects. However, one must not forget to confirm the homogeneity of variances before testing: IAA production levels frequently violate this assumption and should be treated with a non-parametric Kruskal–Wallis test followed by Dunn's post hoc test. Discrete traits can be easily compared with the chi-square statistic, with multiple layers and residue analysis used to single out the conditions and traits that occur outside expected values. Finally, plot multivariate statistics such as CatPCA (Categorical Principal Component Analysis) and MCA (Multiple Correspondence Analysis) can greatly facilitate simultaneous visualization of the multiple correlations between PGP traits, bacterial taxa, and environmental conditions, as long as numeric, ordinal, nominal, and supplementary variables are treated as such. For more information on multivariate statistics for microbial ecology, see Ramette (2007).

Many research institutes worldwide have standing long-term trials involving different fertilization conditions at experimental stations, which are perfect for identifying these interactions and which greatly facilitate studies that take this type of statistical approach. A single practiced microbiologist who knows what comparisons must be made can isolate, screen, and analyze the PGP traits of hundreds of isolates in just 6 months, which also facilitates the studies that take this type of approach. Given the relative ease and plentitude of opportunities to run this comparison, the intense biological interaction of PGPR with fertilizers, and the importance of this issue with respect to resource conservation and economic sustainability, additional research on this topic that takes this approach is greatly encouraged.

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Section 17

Field Studies, Inoculum Preparation, Applications of Nod Factors

Chapter 96

Appearance of Membrane Compromised, Viable but Not Culturable and Culturable Rhizobial Cells as a Consequence of Desiccation

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96.1 INTRODUCTION AND DISCUSSION

According to Verón et al. (2006), 40% of the world's surface is threatened by desertification-related problems, and consequently the degradation of soil quality due to drought and salinity. Drought and salinity are considered the most important abiotic stresses in many areas in the world, and it is estimated that 1 billion people worldwide populate these lands. Of particular importance to the agricultural industry is the impact of these harsh environmental conditions on the soil-borne endogenous group of proteobacteria, the rhizobia (Zahran, 1999; Fierer et al., 2003; Griffiths et al., 2003; Dardanelli et al., 2012).

The *Rhizobiaceae* is a bacterial family of enormous agricultural importance due to their ability to fix atmospheric nitrogen in an intimate relationship with plants in root or stem nodules enhancing growth under nitrogen-limiting conditions. This relationship is negatively impacted by drought-related stresses (Jones et al., 2007; Zahran, 1999). In addition, these bacteria can also improve drought tolerance of agricultural crops (Grover et al., 2011; Zahran, 2010; Dodd and Pérez-Alfocea, 2012; Bianco and Defez, 2009). To make optimal use of the process of nitrogen fixation, seed inoculation companies apply *Rhizobium* strains to the seed surface that are selected for their ability to efficiently colonize the rhizosphere and fix nitrogen. Unfortunately, many inoculants remain unreliable because of the inability of bacterial cells to persist under adverse conditions, negatively affecting colony-forming units (CFU) of added rhizobia (Kosanke et al., 1992; Deaker et al., 2005; Catroux et al., 2001; Smith, 1992; Bullard et al., 2004; Herridge, 2007; see Chapter 97). Furthermore, Ilyas and Bano (2012) provided a nine-point list of characteristics that plant growth-promoting rhizobacteria (PGPR) should have for successful dry-base formulation development. This list includes desiccation resistance.

The ability of selected strains to survive desiccation depends on many factors, for example, the drying method used, such as forced-drying using vacuum versus air-drying, the media used, the speed and severity of drying, the extent and speed of rehydration, the growth phase, the drying temperature, the availability of solutes, and the carrier material (Vriezen et al., 2006; 2007). In addition, intragenic

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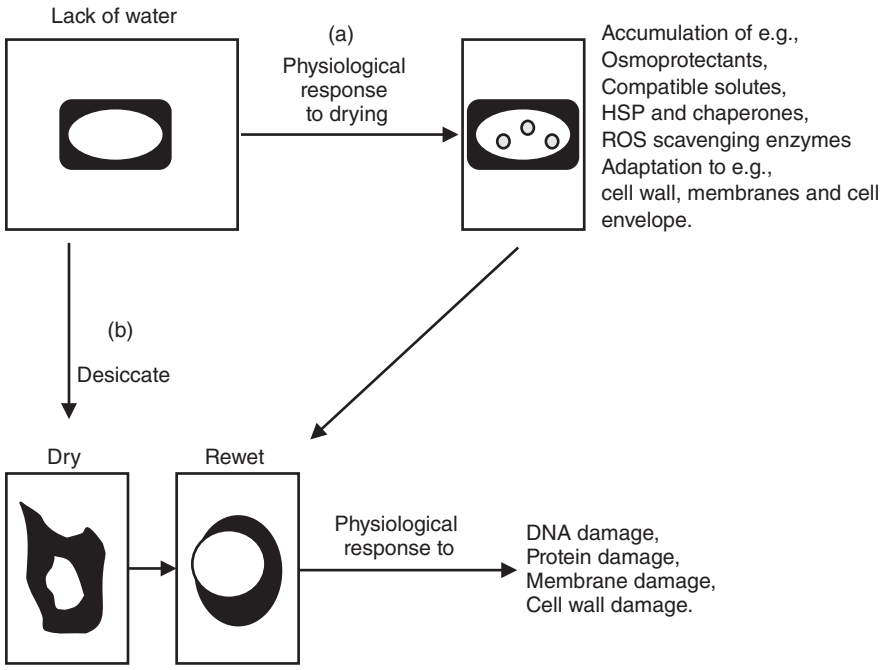

Figure 96.1 Model representing two hypothetical pathways for responses of rhizobia to desiccation stress and desiccation-induced damages. The "preceding-storage induction" pathway (a) implies a response to water, osmotic, or salt stress, and the "post-storage induction" pathway (b) implies a response to the desiccation-induced damages upon rewetting. Reprinted with permission from Vriezen et al. (2007).

differences to cope with desiccation stress affect survival. For example, slow growers tend to be more desiccation resistant than fast growers (Zahran, 2001). These data suggest that no single trait affects the ability of rhizobia to survive desiccation but that several mechanisms are likely responsible (Vriezen et al., 2006; 2007; 2013).

In a review by Vriezen et al. (2007), the authors provided a hypothetical model for the response of rhizobia to desiccation (Fig. 96.1). The model includes two not mutually exclusive physiological responses. Upon drying, rhizobia sense the consequences of drying, which may be the lowering of water activity (A_w), accumulation of solutes, and concentration of enzymes. Thus far it remains unclear as to how desiccation is sensed and resistance is mediated (Vriezen, 2005; Vriezen et al., 2007; Hirsch, 2010; see Chapters 39, 40). The response likely includes the accumulation of osmoprotectants and compatible solutes known to increase desiccation survival. For example, trehalose increases the ability to survive desiccation (McIntyre et al., 2007). In addition, heat shock protein (HSP) and chaperones and reactive oxygen species (ROS) scavenging enzymes accumulate (Feng et al., 2002; Cytryn et al., 2007). In contrast, when cells are desiccated, cells are not active; thus, damage accumulates due to the inability to repair them. Consequently, upon rewetting and regaining of metabolism cells get the opportunity to repair damage accumulated during storage or that acutely appears upon rewetting. These damages include damage to DNA, and damage to membranes and the cell wall (Humann et al., 2009; Potts, 1994; Leslie et al., 1995; Vriezen et al., 2012; Salema et al., 1982; Bushby and Marshall, 1977b; see Chapter 39).

Although improvement of long-term survival and seed inocula storage time has been the focus of desiccation research, relatively little work has focused primarily on the bacterial cell. Most, if not all, research only used culturability as a measure to estimate survival consequently marginalizing often the vast majority of cells not forming colonies. However, what is the fate of the cells not forming colonies? Recently, Vriezen et al. (2012) described the appearance of viable but not-culturable (VBNC) *Sinorhizobium meliloti* cells upon desiccation and resuscitation. Because a major target for desiccation stress is the cell membranes, they hypothesized that cells, which lost the ability to form colonies, have compromised cell membranes (Potts, 1994; Leslie et al., 1995). Thus, it was expected that all cells not forming colonies were membrane compromised. To test this, Vriezen et al. (2012) applied the live/dead stain to cells after desiccation. This stain uses two dyes, syto-9 and propidium iodide, which differ in their ability to cross the cytoplasmic membrane. Syto-9 can always cross the membrane and stains all cells green. However, propidium iodide can only cross the membrane when the permeability is increased and stains the cell red. These red cells are dead. When this stain was used on rhizobial cells prior to and after drying, unexpected results were obtained. The data presented in Figure 96.2 shows that two staining methods (crystal violet and live/dead) yielded the same results prior to drying, after 3 days at 100% RH and after 3 days at 22% RH. The increase in countable cells at 100% RH conforms to what one expects since an increase in colony-forming units was observed over 3 days at 100% RH in previous studies (Vriezen et al., 2006). However, a change in the

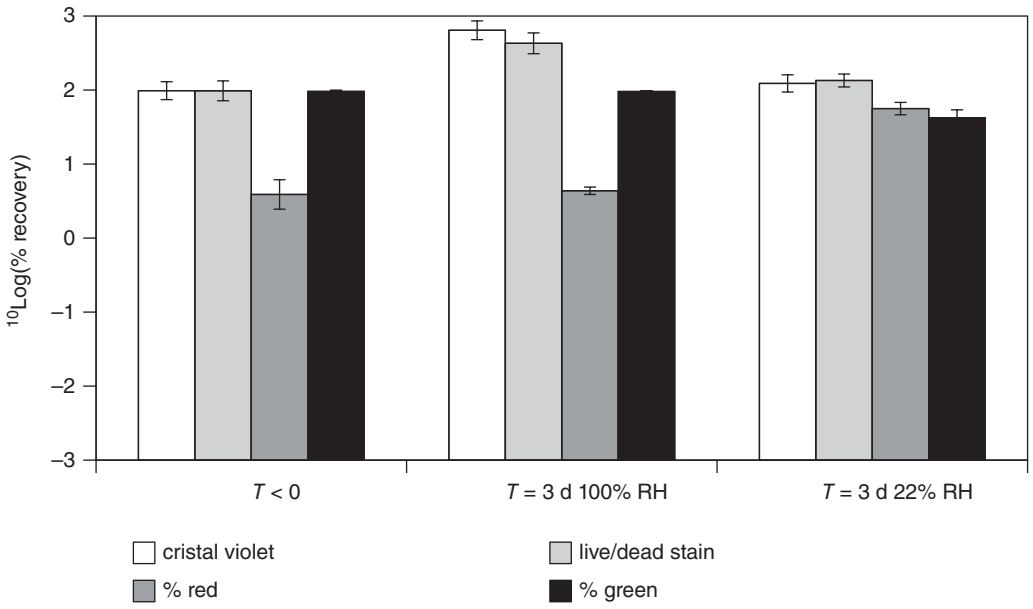

Figure 96.2 Survival and recovery of *S. meliloti* cells after drying and rewetting. Quantitative recovery of cells (direct count) after 3 days of storage under 100% or 22% RH on nitrocellulose filters (white bar = cristal violet, light grey bar = Live/dead, dark grey bar = red%, black bar = green%). All error bars represent the SEM. Reprinted with permission from Vriezen et al. (2012).

fraction of red (dead) and green (living cells) was observed. Prior to drying, the red fraction of cells was very low ($4.4 \pm 0.5\%$) and increased substantially during desiccation to $56.9 \pm 10.6\%$. During this process, culturability decreased to 3.1%. If it is assumed that the colony-forming cells are a fraction of the viable cells, we can only conclude that many cells are alive but are not able to form colonies and are in a VBNC state. This VBNC state exists in many microorganisms, including rhizobia (Manahan and Steck, 1997; Alexander et al., 1999; Basaglia et al., 2007; Räsänen et al., 2001; Vriezen et al., 2012; Catroux et al., 2001). The induction of this physiological state by desiccation is a novel and very relevant observation since only cells able to form colonies can infect plants, as reported for strain *S. meliloti* 41 (Basaglia et al., 2007). The term VBNC applies directly to the observations presented in Figure 96.2: Cells of *Sinorhizobium meliloti* 1021, which were rehydrated after desiccation, can be divided into three different fractions of cells after desiccation, the membrane-compromised (MC), the viable but not culturable (VBNC), and the culturable cells (colony-forming units, or CFU) and correspond to fraction III, II and I in Vriezen et al. (2012) respectively.

96.2 THE MEMBRANE-COMPROMISED (MC) FRACTION

96.2.1 Fraction III

That cell membranes are a target for desiccation is not novel; however, the extent to which desiccation compromises membranes in *Sinorhizobium meliloti* 1021 is unexpected. In the aforementioned study, $56.9 \pm 10.6\%$ of cells stain red and have lost membrane integrity, indicating that of the very substantial number of cells not forming colonies ($100 - 3.1\% = 96.9\%$), 59% have nonfunctional membranes. Thus, the loss of membrane integrity is the main cause of death for *Sinorhizobium meliloti* 1021. Vriezen et al. (2012) hypothesized that this desiccation-induced loss of membrane integrity can be explained by changes in phase transition of phospholipid membranes due to the removal of water affecting the phospholipid head spacing and due to rehydration and the consequent breakage of the cell wall. Furthermore, lipid peroxidation and Fe^{3+}-catalyzed oxidation also lead to the loss of structure of the membranes when cells are not able to repair damages (Deaker et al., 2004; Potts, 1994). Under normal wetted conditions, membranes are in the liquid crystalline phase (Fig. 96.3a). Upon the extraction of water phase transition occurs leading to the gel-phase of the membrane. Upon rewetting, phase transition occurs again, leading to leakage of cell constituents from the cell and to the loss of membrane integrity and cell death.

Phase transition can be followed using Fourier Transformation Infra-Red (FTIR, Leslie et al., 1995) spectroscopy in which the vibration frequency of the phospholipid head groups is measured (Fig. 96.3b). Upon decreasing temperatures, this frequency decreases, indicating membrane transition. The temperature at which this transition occurs is the midpoint temperature. Interestingly, if ambient temperature is higher than the membrane midpoint temperature, no phase transition occurs upon drying and rewetting, reducing cell death. However, when drying takes place at an ambient

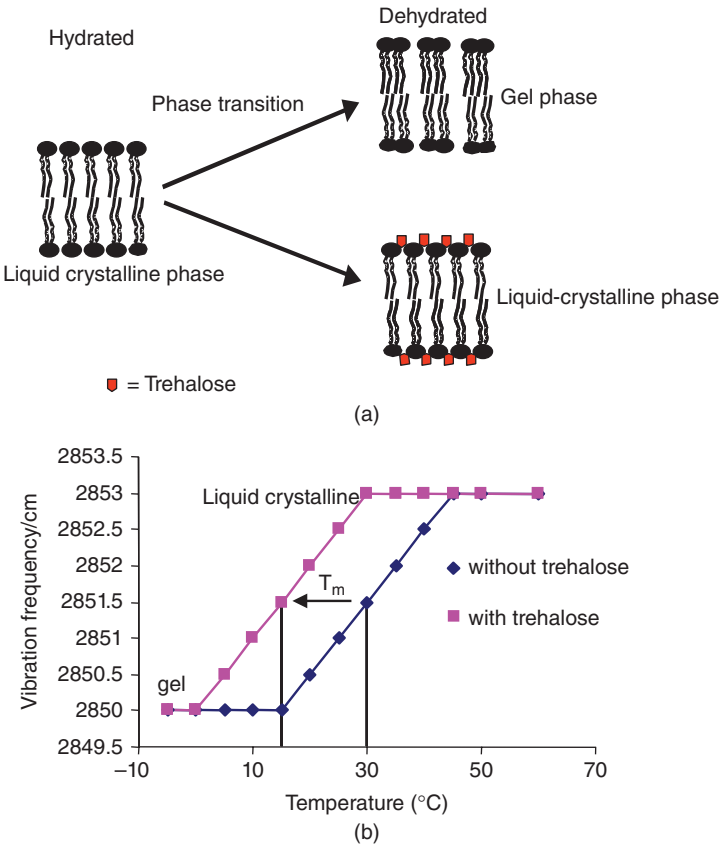

Figure 96.3 Membrane properties: (a) Phase transition from liquid crystalline to gel phase and prevention by trehalose (amended from Welsh, 2000). (b) Vibration frequencies of the phosphate head groups and the effect of trehalose on the midpoint temperature (Tm) (amended from Leslie et al., 1995).

temperature below the midpoint temperature, cell death due to membrane transition is increased. A consequence of a change in membrane midpoint temperature can be seen in the change in vibration frequency (Fig. 96.3b). When the midpoint temperature is lowered one expects the temperature at which phase transition occurs to be lower, resulting in a wider window of ambient temperatures higher than the midpoint temperature, increasing survival. Survival data indicates that this is what happens after desiccation of *Sinorhizobium meliloti* 1021. An increase in viability was observed with increasing temperature with a maximum at 37 °C, indicating that this process may underlie the phenomenon (Vriezen et al., 2006).

The structural adaptations to membrane phospholipids that affect fluidity and midpoint temperature are the following: nonreducing sugars such as trehalose stabilize the phospholipid head spacing of the membranes, leading to a decrease in the membrane midpoint temperature and increased survival even at lower ambient temperatures (Fig. 96.3a and b). In addition, longer fatty acids decrease fluidity leading to an increase in the midpoint temperature. Furthermore, an increase in *cis*-bonding, and thus an increase in unsaturation, leads to increased fluidity, and a lower midpoint temperature (Leslie et al., 1995). Therefore, a decrease in the unsaturated/saturated (u/s) ratio of membrane phospholipids leads to a lower fluidity and a higher midpoint temperature. Boumahdi et al. (1999) studied survival after desiccation of *S. meliloti*, *B. japonicum*, and *B. elkanii* in relation to the growth-phase and the fatty acid u/s ratio. Even though differences in u/s were found depending on the growth phase, these differences did not correlate with the ability to survive desiccation at many RHs (3–83.5% RH) except under the following conditions: In *B. elkanii*, an increase in saturation leads to a decrease in desiccation survival at 67.8% RH at 30 °C, and in *B. japonicum*, the same was seen at 3% and 22%RH. While the expected correlations were seen in bradyrhizobia under certain conditions, this was not seen in *S. meliloti* RCR2011. In another publication by Boumahdi et al. (2001), growth at decreased water activities (A_w) affected the u/s ratio in *S. meliloti* 2011, *B. elkanii*, and *B. japonicum*. This effect was strongest in *B. elkanii*. Surprisingly though, with decreasing A_w a decrease in u/s ratio was found, counter-intuitive to what one would expect in order to survive water stress with the membrane as major target. Why these correlations were not observed across the range of A_w's, RHs, strains, and growth phases tested is unclear. However, it indicates that other mechanisms underlie these phenomena.

In addition to the responses described above, hypothetically, an increase in the concentration of hopanoids

should increase fluidity and lower the midpoint temperature. Hopanoids are the prokaryotic equivalent of cholesterol in eukaryotes (Kannenberg and Poralla, 1999; Kannenberg et al., 1995). Their function remains unknown; however, their presence in membranes leads to reduced permeability and increased order of membranes above the midpoint temperature at which molecular disorder threatens membrane stability. These aliphatic compounds have also been identified in *Rhizobium* but to our knowledge have not yet been studied in relation to desiccation survival.

96.3 THE VIABLE BUT NONCULTURABLE (VBNC) FRACTION

96.3.1 Fraction II

The second most important fraction in a culture of cells after desiccation is formed by the VBNC cells. Many environmental factors have been identified inducing a VBNC state in bacteria, such as temperature stress, osmotic upshift, and oxygen stress, tap water and the VBNC inducing component copper in *A. tumefaciens* and *R. leguminosarum* (Oliver, 2005; Räsänen et al., 2001; Manahan and Steck, 1997; Alexander et al., 1999). Moreover, desiccation can induce a VBNC state in *E. cloacae* and *S. meliloti* (Pederson and Jacobsen, 1993; Vriezen et al., 2012). This VBNC fraction can be divided into two subfractions, those cells for which VBNC is reversible and can be resuscitated (temporarily nonculturable), and those for which VBNC is a permanent state (permanently nonculturable) (Maraha, 2007). In a paper by Hammes et al. (2011), the authors named these two fractions "potentially reversible, starved or injured" and "irreversible, or dying/dead". The demarcation of these two fractions is a hard to assess amount of DNA and protein damage. It remains unclear as to which level of damage leads to death or the inability to resuscitate.

Several researchers have attempted to understand the conditions modulating the culturability of bacteria. Barry et al. (1956) noted that autoclaving media leads to an increase in H_2O_2, decreasing CFUs. The addition of sodium pyruvate and catalase to the medium can increase resuscitation in many organisms (Mizunoe et al. 1999; Imazaki and Nakaho, 2009). However, this approach proved unsuccessful in *S. meliloti* 1021 and 41 (Basaglia et al., 2007; Vriezen et al., 2012). It appears that all desiccation- and O_2 limitation-induced VBNC cells are in a permanent state of nonculturability under the conditions tested (Basaglia et al., 2007; Toffanin et al., 2000). In contrast, a very slow supply of oxygen could resuscitate some cells (Basaglia et al., 2007). These results indicate that resuscitation from the VBNC state differs between *E. coli* and *Rhizobium*, even though O_2 damage may occur in both cases.

One explanation for the occurrence of desiccation-induced rhizobial VBNC cells is that these cells are without a functional template for the replication of DNA but have intact membranes. DNA is a major target of desiccation in microorganisms inducing double strand breaks in *E. coli* and *D. radiodurans* (Asada et al., 1979; Mattimore and Battista, 1996). In support of this hypothesis are the observations by Humann et al. (2009), who isolated a desiccation sensitive *Sinorhizobium* mutant with a Tn*5* insertion in its *uvr*C locus, which is involved in DNA repair (see Chapter 39). Therefore, the inability to repair desiccation-induced DNA damage leads to a decrease in CFUs and likely to an increase in VBNC cells in rhizobia.

To identify additional physiological responses potentially involved in the VBNC state of rhizobia, we consulted three studies addressing the physiological responses of the VBNC state in *Pseudomonas* and *E. coli*, both proteobacteria. The proteins identified in these three studies are summarized in Table 96.1 (Maraha, 2007; Asakura et al., 2008; Muela et al., 2008). Several loci, OmpW, HisJ, and ProX, were found in both proteobacteria in more than one study. Surprisingly, OmpW, found to be strongly expressed in VBNC cells in several microorganisms and studies, could not be identified in *S. meliloti*. Actually, only HisJ has significant identity in *S. meliloti* 1021 (>95% of the query sequence, and >30% identity). Using the same criteria, five more loci were identified and are DdpA, TpiA, LeuD, OppA, and EF-TU, which, together with HisJ are the first set of *S. meliloti* candidate loci affecting the VBNC state. Interestingly, neither the loci identified by Humann et al. (2009) nor those involved in trehalose metabolism (Reina-Bueno et al., 2012; McIntyre et al., 2007; Flechard et al., 2010) or those responsive upon desiccation (Cytryn et al., 2007) were identified (see Chapter 39), indicating that DdpA, TpiA, LeuD, HisJ, OppA, and EF-TU represent a novel set of candidate loci for the rhizobial VBNC state.

How do these observations relate to rhizobia? Identification of the proteins mentioned above indicates that damage to amino acid metabolism and protein synthesis may result in permanent VBNC cells in *Rhizobium*. For example, Asakura et al. (2008) showed that HisJ, LeuD, and OppA were increased in an oxidative- and osmo-tolerant *E. coli* strain, while TpiA was decreased compared to an oxidative- and osmo sensitive *E. coli* strain after passing through the GI track. This would indicate that strains not sensitive to oxygenic stress, and thus "VBNC resistant," have increased expression of HisJ, LeuD, and OppA. In Muela et al. (2008), EF-TU (TufA, involved in recruiting charged tRNA to the a-site on the rhibosome) was found expressed in the VBNC state in phosphate-buffered saline (PBS). According to Barcina and Arana (2009), and conforms the findings by Kong et al. (2004) in *Vibrio vulnificus* and Asakura et al. (2008) in *E. coli*. Hydrogen peroxide-sensitive *Vibrio* cells, having lost catalase activity, are entering the VBNC state and have increased OmpW and TufA levels.

Table 96.1 Candidate Loci in *S. meliloti* 1021 Potentially Involved in the VBNC State

Information query sequences*				Information results from blast agains *S. meliloti* 1021 database§						
Gene Name	Organism†	Reference	Query NCBI acc#‡	Score**	E-value	Query %	Identity %	Locus	Gene Name	Description
ddpA	Pf	Maraha	YP_002870490	1349	$1.7e^{-176}$	97	50	Smc00786	*dppA1*	Oligopeptide ABC transporter
hisJ	Pf/Ec	Maraha/Asakura	AP_002909	472	$7.7e^{-45}$	99	42	Smc00140		Putative amino acid binding protein
livK	Pf	Maraha	YP_002870995	231	$4.6e^{-15}$	96	24	Smc02355		Putative branched chain amino acid binding ABC transporter
proX	Pf/Ec	Maraha/Asakura	AP_003252	209	$1.5e^{-10}$	96	26	Smc00672	*hisX/hutX*	Histidine ABC transport
ompW	Pf/Ec	Maraha/Asakura/Muela	AP_001882	*NI*						
pstI	Ec	Asakura	P08839	189	$6.2e^{-14}$	36	31	Smc00025	*ppdK*	Putative pyruvate phosphate dikinase
serA	Ec	Muela	NP_417388	319	$1.9e^{-28}$	60	31	Smc02849	*gyaR*	Probable glyoxylate reductase
thrC	Ec	Muela	NP_414545	541	$2.1e^{-49}$	88	33	Smc00077	*thrC1*	Probable threonine synthase
leuD	Ec	Asakura	P30126	517	$9.9e^{-48}$	95	52	Smc03795	*leuD*	Probable 3-isopropylmalate dehydrolase
dps	Ec	Asakura	CAA49169	*NI*						
oppA	Ec	Asakura	P23843	912	$4.4e^{-90}$	97	38	Smb21192	*oppA*	ABC transporter tri/tetra peptides
dnaK	Ec	Asakura	P23869	194	$1.5e^{-15}$	84	39	Smc01700	*ppiA*	Putative peptidyl-prolyl cis-trans isomerase
znuA	Ec	Asakura	P39172	388	$2.6e^{-51}$	61	40	Smc04245	*znvA*	Probable Zinc uptake ABC transporter
tpiA	Ec	Asakura	P04790 (obsolete number)	472	$2.5e^{-43}$	97	42	Smc01023		Triose phosphatase isomerase
tig	Ec	Asakura	P22257 (obsolete number)	596	$4.7e^{-55}$	99	33	Smc02050	*tig*	Probable trigger factor
EF-Tu	Ec	Muela	BAE77952	1559	$7.2e^{-157}$	100	75	Smc01312	*tufA*	Probable EF-Tu

*Query sequence obtained from several studies in related microorganisms (Maraha, 2007; Muela et al., 2008; Asakura et al., 2008).

†Pf = *Pseudomonas fluorescens* SBW25, EC = *Escherichia coli* K12.

‡Where available, the *E. coli* sequence was used and obtained from NCBI.

§Blasted against the *S. meliloti* database http://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi using tblastnt.

**NI = Not identified.

The hypothesis stated above is further supported by the identification of *rel*A mutants with a desiccation-sensitive phenotype by Humann et al., (2009; see Chapter 39). RelA (stringent response) is stimulated by aminotriazole (AT), a histidine analog inducing histidine starvation (Wells and Long, 2002; Krol and Becker, 2011). Histidine starvation induces *rel*A and therefore the stringent response. HisJ mutants potentially induce histidine starvation. These findings support the hypothesis that desiccation induced VBNC cells are affected in amino acid metabolism and translation. A substantial part of the permanently nonculturable rhizobial cells after desiccation may thus be irreversible VBNC, or dying/dead due to substantial amounts of DNA and protein damage.

96.4 THE CULTURABLE FRACTION (CFU)

96.4.1 Fraction I

The CFU fraction is the smallest of the three recognized fractions of rewetted *Sinorhizobium meliloti* 1021 after desiccation. This fraction is so small (3.1%, Vriezen et al., 2012) that it falls within the error of measurement of the MC fraction (±10.6%). Thus, even a doubling in the fraction of culturable cells would not be reflected in a significant change in dead and living cells. It is the culturable fraction that counts in formulations of rhizobia since nongrowing but living cells do not contribute to nodule formation (Basaglia et al., 2007). Therefore, understanding the conditions and cellular responses that increase this fraction remains of crucial importance. Even though no data exist on how osmotic stress and temperature affect the appearance of VBNC and MC fractions, more is known about these conditions in relation to desiccation survival.

96.4.2 Effect of Osmotic and Salt Stress

In a review by Vriezen et al. (2007), the authors hypothesized about the effect of NaCl stress on the ability of *S. meliloti* to survive desiccation. Exposure to NaCl during drying increases the ability to survive desiccation even considering that salt stresses, osmotic stresses, and desiccation stress are very different in essence. Osmotic stress is the abundance of solutes, salt stresses, and of (non) toxic ionic compounds, while desiccation stress results from the lack of water. The reason for the hypothesis is the available data indicating an overlap in response between the stresses reviewed by Vriezen et al. (2007). The conclusions were that (i) chloride stress induces a response in combination with nutrients from the media that lead to an increase in survival, (ii) the response is strain specific and (iii) the increase in CFU during NaCl-mediated desiccation is physiological in origin. Even though the response of *S. meliloti* to NaCl does increase CFU after desiccation, it only excludes some aforementioned stresses from inducing these physiological responses. However, in their review Vriezen et al. (2007) argued that screening for loci responsive to NaCl stress would select loci potentially involved in survival during desiccation. In support of this are the findings by Streeter (2003) that NaCl increases intracellular trehalose content in *Bradyrhizobium japonicum*, and the finding by Humann et al. (2009), who showed that a *rpo*E2 mutant was sensitive to desiccation (see Chapter 30). RpoE2 is a response regulator for envelope stress. The hypothesis is further supported with the identification of the *S. meliloti* 1021 mutant with a Tn*5lux*AB transcriptional fusion inserted in a NaCl-inducible putative open reading frame (ORF, *ngg*) sensitive to survival during desiccation (Vriezen et al., 2005 and Vriezen et al., 2013). Ngg is responsive to NaCl stress and also affects the ability of *S. meliloti* 1021 to survive desiccation.

Which NaCl-mediated responses affect survival during desiccation? We address four potential responses. Firstly, although certain compatible solutes and osmoprotectants accumulate during NaCl and osmotic stress and have a positive effect on the survival during desiccation of *Rhizobium*, others have not. For example, the recently identified NaCl-induced loci *asnO* and *ngg* involved in the production of the dipeptide NAGGN show differential responses to desiccation (Vriezen et al., 2005; Vriezen et al., 2013; Sagot et al., 2010). A Tn*5lux*AB insertion in locus *asn*O does not lead to a decrease in survival during desiccation, while an insertion in locus *ngg* does. This indicates that NAGGN accumulation as a response to NaCl stress does not affect the ability to survive desiccation since both loci are involved in the synthesis of NAGGN. In contrast, compatible solutes such as sucrose and trehalose are known to affect survival by their stabilizing abilities of the cell membrane. Trehalose accumulates in osmo-stressed rhizobia and provides protection against desiccation by maintaining membrane integrity during drying and rewetting (see Chapter 97). Its presence may explain the increase in desiccation survival during the stationary phase and when rhizobial cells are exposed to NaCl (Welsh and Herbert, 1999; Breedveld et al., 1990; 1993; Streeter, 2003; Leslie et al., 1995; Potts, 1994; Reina-Bueno et al., 2012; McIntyre et al., 2007; Flechard et al., 2010). Gouffi et al. (1998; 1999; 2000) found that trehalose and sucrose are synthesized *de novo* during exponential growth. Uptake mechanisms in rhizobia were also described; an *agl* operon for trehalose/maltose and sucrose uptake (*smb03060-03065*) was identified by Willis and Walker (1999), and Jensen et al. (2002) identified an alternative trehalose/maltose/sucrose operon (*thu*, *smb20324-20330*). Domìnguez-Ferreras et al. (2006) showed that the *thu* operon is upregulated during an osmotic

upshift and the importance of the osmotic stress-responsive loci *ots*A and *tre*S in trehalose accumulation. McIntyre et al. (2007) showed that *ots*A provides resistance to desiccation. Interestingly, trehalose synthesis genes (*ots*AB and *tre*S) are increasingly expressed during drying of *Bradyrhizobium japonicum* (Cytryn et al., 2007) and Sugawara et al. (2010) shows that *tre*S and *tre*Y mutants of this organism have lower survival rates after desiccation.

Wei et al. (2004) and Miller-Williams et al. (2006) also identified *Sinorhizobium* mutants unable to grow at increased NaCl concentrations. The mutations causing these phenotypes were traced to genes involved in the central metabolism, such as elongation factors, chaperones, and cell division proteins. Moreover, expression of genes for DNA ligases was higher as was the expression of a putative DNA polymerase, an invertase and a ribonuclease. These observations are most interesting considering VBNC cells may not be able to resume growth after exposure to desiccation conditions due to extensive DNA damage. Whether these responses affect desiccation resistance or the appearance of desiccation-induced VBNC cells in *Rhizobium* remains to be seen.

Polysaccharides are of interest with respect to desiccation since adaptations of the polysaccharide composition have been observed for *S. meliloti* undergoing osmotic stress and are known to affect survival during dry conditions (Breedveld et al., 1990; Lloret et al., 1998; Chenu, 1993). Vanderlinde et al. (2011) identified a *R. leguminosarum* Tn*5* mutant in which exopolysaccharide (EPS; see Chapter 36) production positively correlates with desiccation resistance. The open reading frame mutated is *RL2975*; however, a similar ORF does not exist in *S. meliloti*. The mutant was not sensitive to hyperosmotic stress, or to detergents, suggesting that the outer membrane was not affected. However, it is naïve to consider polysaccharides a panacea to all desiccation-related issues. Vriezen et al. (2007) evaluated many reasons why this is not the case and gave examples of studies resulting in contradictory observations. For example, a decrease in survival of colony-forming rhizobia was observed upon the addition of polysaccharides when dried at a RH > 3%, but an increase in survival at 3% relative humidity (Mary et al., 1986). Polysaccharide-producing variants of *Rizobium trifolii* in sandy soil and under fast drying conditions showed no consistent improvement in survival (Bushby and Marshall, 1977a). Osa-Afiana and Alexander (1982) showed that, when dried slowly in Collamer silt loam, the production of EPS decreases survival during desiccation of *Bradyrhizobium japonicum* strains, even though polysaccharides did increase survival of *R. trifolii* 412 in a Lima silt loam (Pena-Cabriales and Alexander, 1979). The reason for these apparent contradictions is likely due to the complexity of- and ambient conditions during desiccation. A polysaccharide may provide protection under one condition while is detrimental under other conditions. The mechanisms by which polysaccharides provide protection are not clear and specific properties of polysaccharides have different effects on a microorganism's ability to survive desiccation. Four of these properties are (i) buffering against changes in water content, (ii) exclusion of toxic compounds, such as Cl^- and O_2, (iii) the final water content of polysaccharides under ambient conditions, and (iv) the effect of hysteresis in the water retention isoterms of polysaccharides (Potts, 1994; Rinaudo, 2004; Chenu, 1993).

Existing data on the environmental conditions affecting polysaccharide production show that an increase in osmotic pressure results in enhanced production of high molecular weight (HMW) succinoglycan over low molecular weight (LMW) succinoglycan (Breedveld et al., 1990) and that the expression of genes involved in EPSI production are upregulated during salt stress (Rüberg et al., 2003, Jofré and Becker, 2009; see Chapter 36). These observations suggest that in *S. meliloti* NaCl-dependent EPS production leads to the production of HMW succinoglycan, resulting in an increase in CFUs after desiccation. In addition, structural changes under the influence of osmotic and salt stress have also been reported for lipopolysaccharides (LPS) (Bhattacharya and Das, 2003; Lloret et al., 1995). Interestingly, Lloret et al. (1995) found a different LPS content in EFB1 cells grown on different salts, while polyethylene glycol (PEG) 200, which causes only osmotic stress, does not induce such a change. These differential responses may correlate with the differences in survival during desiccation when exposed to several different salts and argue for a potential role of LPS in survival during desiccation (Vriezen et al., 2006). Indeed, Vanderlinde et al. (2010; 2011) and Vanderlinde and Yost (2012) showed that a mutation in the *fab*F1 and *fab*F2 gene in *R. leguminosarum* involved in LPS formation increased sensitivity to desiccation and osmotic stress. Thus, structurally intact LPS are important in protecting *R. leguminosarum* cells against desiccation.

Vriezen et al. (2007) hypothesized that enzymes involved in the production of HMW succinoglycan would positively affect CFUs. For example, mutations in *S. meliloti* ExoP (Smb21506) was found to block polymerization of EPS1, and ExoQ (Smb20944) is required for the production of HMW succinoglycan (González et al., 1998; Jofré and Becker, 2009). In support of this theoretical consideration was the ~5-fold induction of *exo*P in desiccated *Bradyrhizobium japonicum* (Cytryn et al., 2007). Interestingly, in *S. meliloti* depolymerization of HMW leads to the production of LMW succinoglycan, which is ExoK (Smb20955) and ExsH (Smb20932) mediated (York and Walker, 1998). However, Cytryn et al. (2007) did not find these genes in their induction studies.

Lastly, Cytryn et al. (2007) found an upregulation of glycogen synthase (*glg*A) during desiccation. Glycogen may assist in restoring cell volume after osmotic shock (Han et al.,

2005). The *glgA2*, *glgB2*, and *glgX* genes involved in glycogen metabolism (*smb20704, smb21447, smb21446* respectively) are higher expressed during exposure to osmotic stress and may have a role in desiccation survival.

96.4.3 The Impact of Temperature

Theoretically, temperature is involved in survival during desiccation through the phase change of membranes during drying and rewetting leading to the loss of membrane integrity (Leslie et al., 1995). The logical consequences of this process would be that an increase in drying temperature prevents membrane transition. Vriezen et al. (2006) and the authors of this chapter (Fig. 96.4) found a positive correlation between survival and temperature with an optimum at 37 °C. This indicates a potential physiological response to temperature affecting survival after desiccation.

Vriezen et al. (2007) reviewed the conditions in soil and seed inocula and concluded they do not support the *in vitro* observations, because many different researchers obtained contradictory results. They concluded that at least one additional factor must exist applying an unknown, yet overruling stress to dry cells. For example, (i) dry seed inocula have a water activity of 0.45–0.6, and thus still contain a relatively high amount of water (Smith, 1992; Deaker et al., 2004; see Chapter 97). (ii) Isolated rhizobia show large differences in their ability to respond and adapt to life at high temperature which is not necessarily linked to their ability to survive desiccation (Trotman and Weaver, 1995). Therefore, heat-tolerant strains may not have an increased ability to survive desiccation, unless temperature, rather than drought, is the superimposed stress. However, the identification of a *Azorhizobium sesbania* Tn*5* mutant sensitive to drought and temperature reveals a genetic basis for this response in some strains (Rehman and Nautiyal, 2002). In addition, Reina-Bueno et al. (2012) identified an *ots*A mutant of *R. etli* that was affected by drying, but also lost the response to temperature.

The molecular responses to stress in rhizobia were recently reviewed by Alexandre and Oliveira (2012) and include heat-inducible small heat shock proteins (HSP) (Ono et al. 2001; Münchbach et al., 1999), the heat shock proteins DnaKJ, GroESL, and GroEL (Minder et al. 1997; Rodrigues et al. 2006; Rodrìguez-Quiñones et al., 2005; Fischer et al., 1993; see also Chapter 40), transcriptional regulation by RpoH (Narberhaus et al., 2006; Ono et al., 2001), and EPS and LPS (Nandal et al., 2005). Potential sensing mechanisms involve *cis*-acting ROSE elements or RNA thermometers (Waldminghaus et al., 2005; Narberhaus et al. 2006; Nocker et al. 2001), and thermo-induced changes in DNA structure and nucleoid-associated proteins (Shapiro and Cowen, 2012; Steinmann and Dersch, 2013). However, no papers were found on rhizobial thermosensing by responding to changes in cell membranes.

Several of the aforementioned mechanisms may affect survival of rhizobia after desiccation (Cytryn et al., 2007). These authors showed an increase in expression of *gro*ESL-related chaperones, indicating a potential involvement of these genes in survival during desiccation in *Bradyrhizobium japonicum.* It is likely that similar mechanisms exist in *Sinorhizobium meliloti*. Furthermore, Domìnguez-Ferreras et al. (2006) identified several loci responsive to an increase in osmotic and salinity stress also associated with the temperature response. Most interestingly, RpoE2 (Smc01506), affecting survival during desiccation, also controls 44 genes involved in the heat-shock response (Humann et al., 2009; Sauviac et al., 2007; see Chapters 39, 40). Therefore, RpoE2 may regulate the part of the osmotic and temperature response also affecting its ability to survive and grow after desiccation.

96.4.4 NaCl and Temperature: An Interconnected Response to Desiccation?

Intellectually it makes sense that microorganisms respond to an increase of solutes or to temperature in order to respond to desiccation. However, how likely is it to have a molecular junction of a NaCl-inducible gene that, when knocked out, leads to temperature-dependent desiccation sensitivity? In addition to some NaCl-induced loci described earlier, locus *smb01590*, found by Vriezen et al. (2013), also affects survival during desiccation (Fig. 96.4). Interestingly, the ability of the mutant carrying a Tn*5lux*AB insertion in ORF *smc01590* (Sce-1) to survive desiccation is better, albeit not significant ($P = 0.22$), than that of the reference strain at 4 °C. While survival of the reference strain increases with increasing temperature, survival of Sce-1 decreases with increasing temperature, leading to a much better survival of the reference strain at 37 °C. There are at least two different not mutually exclusive explanations for the observation. Firstly, this observation suggests the involvement of the membrane in this process, since the reference strain does respond exactly as explained above: If ambient temperature is higher than the membrane midpoint temperature, no phase transition occurs, reducing cell death and increasing culturability. When drying takes place at an ambient temperature below the midpoint temperature, cell death due to membrane transition is increased.

In mutant Sce-1, it appears that the inability to correctly adjust the membrane midpoint temperature at increased temperature leads to the opposite effect. At 4 °C, cell death in the reference strain and the mutant strain is comparable. At 4 °C, both strains experience an ambient temperature lower than the membrane midpoint temperature, leading to similar survival rates. However, at increased temperatures, the reference strain experiences an ambient temperature comparable or higher than the midpoint temperature, which increases

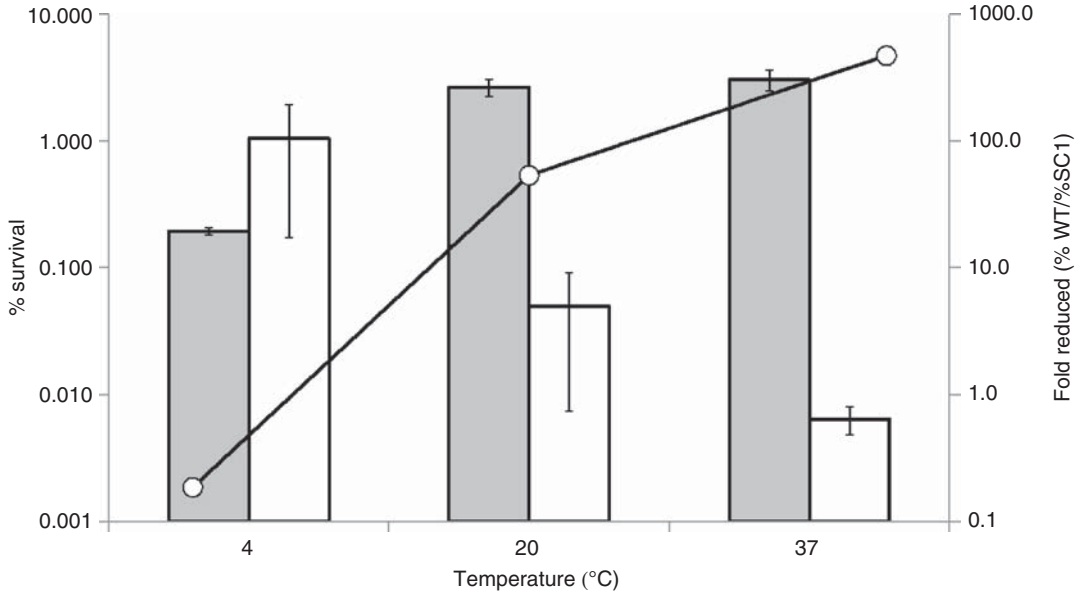

Figure 96.4 Survival after desiccation of *S. meliloti* 1021 (WT, grey bar) and the *smc01590*::Tn*5lux*AB mutant (Sce-1, white bar) and the fold difference in survival of Sce-1 relative to WT (open circle) at 4, 20 and 37 °C.

survival. Owing to the inability to lower the midpoint temperature, the Sce-1 mutant still experiences an ambient temperature lower than the midpoint temperature, leading to a reduced survival compared to the reference strain.

Alternatively, a defect in the temperature response in strain Sce-1 potentially leads to reduced survival rate with increasing temperature. The lack of production of heat shock proteins and chaperones may explain this phenomenon. The postulated nonexclusivity of the two hypothetical explanations allows that one of the responses to NaCl is the decrease in the midpoint temperature.

Interestingly, prodomain analysis of the aminoacid sequence indicates that Smc01590 encodes a 210AA peptide with a leader peptide targeting the cytoplasmic membrane. Smc01590 also contains an SH2/SH3 domain. SH3 domains are called molecular velcro (Morton and Campbell, 1994) due to their ability to form strong bonds with other proteins by targeting proline-rich areas, which are also found in the sequence. Its location and these domain/motif interactions suggest that Smc01590 can form membrane-located proteinaceous structures stabilizing the membrane. Prodomain also predicts several cytoplasmic kinase sites, which are commonly involved in signal transduction directly or indirectly involved in sensing- and maintaining membrane stability. Thus, it appears that Smc01590 is potentially a sensor in a signal transduction pathway, in which changes in membrane fluidity due to temperature and osmotic pressure lead to the expression of downstream loci involved in the lowering of the membrane midpoint temperature. This protein and its locus appear not under RpoE2 control, and may be part of a novel signaling network.

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Chapter 97

Making the Most of High Quality Legume Inoculants

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97.1 INTRODUCTION

In Australia and many other countries around the world, legume inoculants are subject to quality control. The regulatory systems applied vary between countries (Herridge et al., 2002; Bullard et al., 2005). In Canada, Uruguay, and France, quality control is covered by legislation, in the United States it is self-regulated, and in Australia, New Zealand, and South Africa participation is voluntary (see also Chapters 90, 101).

High quality legume inoculants are essential for optimum nodulation and nitrogen fixation by the applied rhizobia but do not guarantee efficacy. Inoculant efficacy is dependent on the rhizobial strain, inoculum potential, survival of rhizobia during delivery to crops, distribution in soil, rhizosphere competency, competition with resident soil rhizobia, and tolerance to unfavorable soil conditions and agrichemicals (Brockwell et al., 1980; Herridge et al., 2002; Deaker et al., 2004; Howieson and Ballard, 2004; Denton et al., 2013).

Adoption of inoculation is dependent on the same conditions that apply to other technologies. Some of the conditions for adoption of technology listed by Guerin and Guerin (1994) include complexity, integration into farming practices, and observable outcomes. The decision to inoculate and the choice of inoculant formulation are largely dependent on convenience and ease of use. Farmers often determine the need to inoculate based on crop history, soil conditions, and inoculation success (Herridge et al., 2002; Denton et al., 2013).

Inoculants are available as either solid or liquid formulations. These different products provide flexibility in application and can be distributed to crops either via the seed or directly to the soil. In Australia, peat-based inoculants are most commonly used.

Granular, freeze-dried, and liquid inoculants are also available but less widely adopted. Peat inoculants are applied in most cases to seed; however, this can be a laborious and difficult task and the market for commercially inoculated or preinoculated seed has grown considerably in the last decade.

In this review, we discuss the quality of Australian legume inoculants and their potential efficacy in relation to both established standards and application techniques (see also Chapter 101). In particular, limitations to efficacy of legume inoculants are highlighted and opportunities for future research are identified.

97.2 WHAT IS INOCULANT QUALITY?

Legume inoculant quality can be defined by a combination of several factors. Factors contributing to peat inoculant quality were described by Roughley and Pulsford (1982) and form

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Table 97.1 Current numerical standards for australian legume inoculants

Product	Count		Expiry (months from date of testing)
	Fresh	Expiry	
Peat (cfu/g)	$\geq 1 \times 10^9$	$\geq 1 \times 10^8$	12–18
Liquid (cfu/mL)	$\geq 5 \times 10^9$	$\geq 1 \times 10^9$	6
Granules (MPN/g)	$\geq 1 \times 10^7$	$\geq 1 \times 10^6$	6
Freeze dried (cfu/vial)	$\geq 1 \times 10^{12}$	$\geq 5 \times 10^{11}$	6

Numerical standards for CB376 for *Lotononis bainesii* are 2×10^8 cfu/g moist peat (2×10^7 cfu/g at expiry). Contaminants in peat, liquid, and freeze-dried products should be absent at 10^{-6} dilution when counted according to ALIRU protocols. Colonies are reacted against appropriate antisera to test for strain trueness and RAPD PCR profiles are randomly checked. Moisture potential of five packets of peat must not be lower than $-\log_{10}$ 4.8 Pa. Batches may be re-submitted within 4 weeks of expiry and expiry extended for a further 6 months if criteria are met. Standard for liquids are based on 3 l bottle used to treat 1 tonne seed. Standard for freeze-dried based on vial is used to treat 1 tonne seed. (Information on standards from Gemell, Hartley and Hartley, 2007)

the basis for quality control tests carried out in Australia by the Australian Inoculant Research Group (AIRG, formerly ALIRU, AIRCS and U-DALS). They include the following:

1. The number of rhizobia per gram of peat carrier
2. Effectiveness of rhizobial strains with intended hosts
3. Moisture content*
4. Freedom from contamination*.

* Important to determine confidence in shelf life.

In addition to these, Lupwayi et al. (2000) lists formulation that is effective and easy to apply, adequate packaging, and clear labeling with instructions for application as important determinants of quality. However, emphasis is given to numerical standards in most quality control systems.

97.3 DEVELOPMENT OF LEGUME INOCULANT STANDARDS

Inoculant standards are set on the basis of the number of viable rhizobia applied to seed. For practical purposes the number is often expressed as the number of rhizobia per gram of inoculant product and therefore application rate must be considered when standards are calculated. The standard for moist peat cultures, of 10^9 cfu/g, was calculated based on a retail standard of 10^8 cfu/g with a multiplication factor of 10, which reflected an average death rate reported by R. Date and R. Roughley in their Masters of Science Theses [R. Roughley, pers comm.]. The retail standard was calculated as the number of rhizobia per gram of peat required that would result in the minimum standard of 300 rhizobia per seed when applied at the manufacturer's recommended rate (Roughley, 1964; Date, 1970).

Ultimately, it is the agronomic performance and number of rhizobia per hectare that is important. However, efficacy is not the same at all sites because of the great diversity of environmental, biotic, and edaphic conditions and although universal standards are applied to inoculants they are agronomically unrealistic. Without adequate practical means of determining the microbiological requirements at each site, standards are based largely on the maximum possible inoculum potential for each formulation.

In the *Rhizobium* Newsletter (1964), Roughley suggested an increase in inoculant standards from 100 rhizobia per seed to 300 rhizobia per seed. Soon after, the minimum standard was increased to 1000 per seed. These changes occurred as a result of improved manufacturing technology. Roughley and Pulsford (1982) commented that research to improve inoculant quality tended to slow when standards could be reached easily and for that reason standards should be under constant review.

Quality standards for inoculant products are usually established to represent a compromise between theoretical and practical possibilities. The current standards for legume inoculants in Australia are listed in Table 97.1 (see also Chapter 101).

97.4 QUALITY AND SHELF LIFE OF AUSTRALIAN LEGUME INOCULANTS

Roughley and Pulsford (1982) stated that the shelf lives of inoculants should be calculated from data collected at the point of sale as temperature during storage and handling may vary widely. Shelf life is dependent on a number of factors including packaging, carrier material, strain, and storage conditions during distribution (Thompson, 1991; Hartley et al., 2005). Most inoculants should be stored at cool temperatures, preferably with refrigeration; however, some strains are better suited to storage at higher temperatures (e.g., 26 °C for some tropical bradyrhizobia).

Table 97.2 Summary of point-of-sale peat inoculant data collected by ALIRU from 2004–2008 (see also Chapter 101)

Species	Total no. packets	Age not known	No. of packets failed			Proportion contaminated (%)	Proportion low count (%)
			Contaminated	Low count + contaminated	Low count		
Annual medic	29	5	4	—	—	13.8	0
Chickpea	64	28	8	1	—	14.1	1.6
Cowpea	66	2	12	3	—	22.7	4.5
Faba bean	86	9	15	1	3	18.6	4.7
Lucerne	134	15	19	—	—	14.2	0
Lupin	137	45	30	6	3	26.3	6.6
Field pea	128	35	12	2	—	10.9	1.6
Pigeon pea	71	—	7	—	7	9.8	9.9
Sub. clover	109	13	14	—	6	12.8	5.5
White clover	49	7	3	2	8	10.2	20.4

97.5 QUALITY OF PEAT INOCULANTS

Australian legume inoculants were collected from retail outlets between 2004 and 2008 and quality was determined as part of an ongoing survey by AIRG (then ALIRU). A summary of the data from peat inoculants is presented in Table 97.2 including the total number of inoculants tested, the number of inoculants for which age was not known, and the number of inoculants that failed due to substandard rhizobial numbers; inoculants that were contaminated with high numbers of non-rhizobial organisms and those that had both substandard counts and contamination.

Contamination in retail samples occurred consistently across inoculant groups and the percentage of contaminated batches ranged between 9.8 and 26.3% (see also Chapter 101). This indicates either that contaminants are systematically introduced during injection or more likely that gamma irradiation is unable to completely eradicate contaminant organisms. A much smaller percentage of inoculants failed because of low numbers. No lucerne or annual medic inoculants and only 1.6% of chickpea and pea/field pea inoculants failed due to low numbers. Five percent of faba bean, cowpea, and subterranean clover inoculants fell below the numerical standards. By far, the highest failure rate was with white clover at 20.4%.

Data from retail surveys were compared with data from the same peat batches submitted to ALIRU at the point of manufacture in Figure 97.1. The data are presented as box plots to illustrate data distribution.

Comparison of the median, range, and distribution of rhizobial numbers at the point-of-sale (retail) batches of peat inoculants and counts from the corresponding batches at the point of manufacture (fresh) indicate a degree of disparity between the two survey points, which varies with strain. Numbers of rhizobia in inoculants for chickpea and lucerne were the most stable with the least disparity between measured parameters. The data for fresh and retail batches had a comparable distribution and only a small drop in numbers. The greatest disparity between survey data was observed with inoculants containing *Rhizobium* spp. (faba bean, pea, and clovers), indicating relative instability of this genus in peat stored in the retail environment. Rhizobial numbers in inoculants containing *Sinorhizobium* and *Bradyrhizobium* were more stable. However, retail data for lupin inoculant indicated high variability of bradyrhizobial numbers.

The reason for disparity between fresh and retail batches is most probably related to variable storage conditions. Survival of rhizobia in peat is high when peat is stored continuously at 4 °C, indicating the potential for long shelf lives (Herridge et al., 2014). However, these results suggest that this is not the case when peat inoculants are distributed. Roughley et al. (1995) found that strains of *Rhizobium* spp. were more sensitive to temperatures between 30 and 40 °C than *Bradyrhizobium* spp. and *Sinorhizobium* spp. strains that were affected at 40 °C. Fluctuations in temperature between 40 and 25 °C resulted in a general decline in numbers over time and survival was dependent on the amount of time stored at each temperature. Peat inoculants in this survey were collected from various conditions including from cool warehouses and sheds, at ambient temperatures in retail shops some of which were air conditioned, refrigerators, and cool rooms. Clearly, the provision of ideal storage temperatures during distribution is a major constraint to assurance of peat inoculant quality and shelf life.

Several inoculants obtained on retail surveys were beyond expiry (see also Chapter 101). However, even when data from inoculants outside the expiry date were removed from analysis, some of the faba bean and clover inoculants still had numbers below the minimum standard. In the case of white clover inoculants, more than 25% [9] of samples were below the minimum standard within the 12-month shelf life. Fewer subterranean clover (1) and faba bean (3) inoculants were below $\log_{10}$ 8.0 inside their shelf lives. There were more samples with low counts when data from

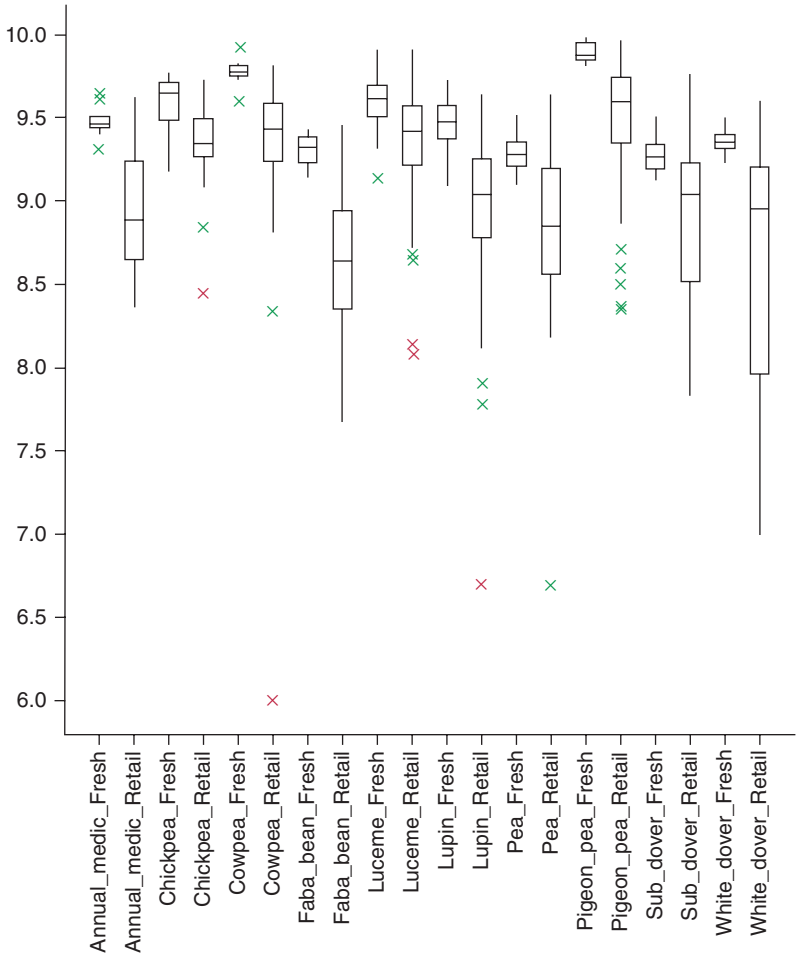

Figure 97.1 Box plot of $\log_{10}$ transformed number of rhizobia per gram of peat inoculant counted at the point of sale (Retail) and the number at the point of manufacture (Fresh) corresponding to the same batch with data outside expiry removed.

inoculants beyond expiry were included, reinforcing the importance of expiry dates for inoculants.

Variability in numbers during storage of legume inoculants is species/strain dependent rather than resulting from variable production practices. It is evident that the relative decline in numbers coupled with inherent strain variability in tolerance to fluctuations in storage conditions should be considered when determining confidence in shelf life of inoculants.

97.6 QUALITY OF GRANULAR AND FREEZE-DRIED INOCULANTS

The current minimum standard for granular inoculants is 6.0 $\log_{10}$ cfu/g at expiry. Point-of-sale data indicated that inoculants prepared from granulated peat had high numbers with a mean of 7.3 $\log_{10}$ cfu/g, a minimum of 6.0 $\log_{10}$ cfu/g, and a maximum of 8.8 $\log_{10}$ cfu/g. The numbers in drier, clay-based granules were generally lower, with a few reaching the minimum standard. The conditions in commercially available granular formulations are considerably different from those in moist peat. Generally, as granular carriers are not pre-sterilized, high numbers of rhizobia obtainable in moist peat culture are difficult to achieve and rhizobial quality of granular inoculants is variable as a result. The change in quality between manufacture and point of sale was not determined for granules as granular inoculants are not submitted to AIRG at the point of manufacture and expiry dates are not always displayed on labels (see also Chapter 101).

One of the major advantages of freeze-dried and liquid inoculant formulations is the reduced risk of contamination. Freeze-dried products have the additional advantage that long-term survival is usually good whereas liquids tend to have a shorter shelf life. Australian freeze-dried inoculant products maintained good survival of rhizobia during storage up to 648 days [1.8 years] for faba bean, 446 days (1.2 years) for lupin, and 557 days (1.5 years) for chickpea with little indication that the numbers were declining. This suggests that extension of shelf life could be possible for freeze-dried inoculants. While there was some variability in rhizobial numbers, the effect of variation in storage conditions in retail outlets was less evident for freeze-dried inoculants than for peat inoculants (data not shown). As variation was consistent from production through storage it

is probable that the main source of variation occurs during production.

97.6.1 Variable Shelf Life

For quality reasons, AIRG distributes authenticated "mother cultures" for each legume species to industry every year. Thus, for most legume species, the shelf life of their respective inoculant is given 12 months (one season). However, the shelf life of peat inoculants for lupin and chickpea (Hartley et al., 2005), faba bean/lentil and field pea (Gemell, Hartley and Hartley, 2003, 2004) was extended from 12 months to 18 months after consideration of several factors including survival of rhizobia within the inoculant over time; survival on seed, and field performance. Extension of shelf life beyond the current growing season has financial benefits for industry as near end-of-season inoculants are still of high quality at the start of the following season. Freight costs are significantly reduced when inoculants can be stored for 18 months. However, there are several reports in the literature of changes to rhizobial cell morphology and physiology during storage in peat (Dart et al., 1969; Feng et al., 2002; Casteriano et al., 2013) and liquid (Maurice et al., 2001), which may have implications for effective nodulation. As agronomic performance is the ultimate test of rhizobial efficacy, it is suggested that any consideration of extended shelf life of inoculants should be supported by the appropriate field data.

97.7 CONTAMINATION IN LEGUME INOCULANTS

Roughley and Vincent (1967), published a manuscript that resulted in widespread improvements to the quality of legume inoculants. The source of peat, moisture content, pH, and choice of neutralization agent and drying temperature were important factors. They also concluded that the high numbers of rhizobia were best supported in peat that had been previously sterilized, preferably by gamma irradiation. In all studied cases, gamma-sterilized peat sustained the highest numbers of rhizobia over time.

Boonkerd (1991) presented data for rhizobial numbers in both sterilized and unsterilized peat in Thailand, which was stored at different temperatures. He found that there was an interaction between sterility, storage temperature, and survival and that this varied between strains. Numbers of both peanut and cowpea rhizobia were predominantly affected by peat sterility whereas storage temperature had a greater effect on soybean rhizobia with better survival at 10 °C regardless of sterility. Generally, inoculants in Thailand prepared using sterile peat are assigned a longer shelf life and lower application rate because of higher numbers.

In the quality control laboratory at AIRG, growth of rhizobia on yeast–mannitol agar is often observed to be inhibited by the presence of contaminants. Inhibition of growth of *Rhizobium leguminosarum* bv. *trifolii* by *Pseudomonas aeruginosa* has also been reported by Olsen et al. (1994b). It is clear from these results that many of the contaminants found in peat produce metabolites that are bacteriostatic toward rhizobia (see Chapter 101).

Data on contaminated inoculants collected in this point-of-sale survey indicate that high levels of contamination had a significant effect on rhizobial numbers ($P < 0.001$) when compared with medium, low, and no contamination (Fig. 97.2; see Chapter 101). When moist peat is exposed to fluctuations in temperature, growth may be encouraged to a point where other contaminant organisms may be favored. Growth of rhizobia occurred when moist peat inoculants were returned to 25 °C after a period of elevated temperature

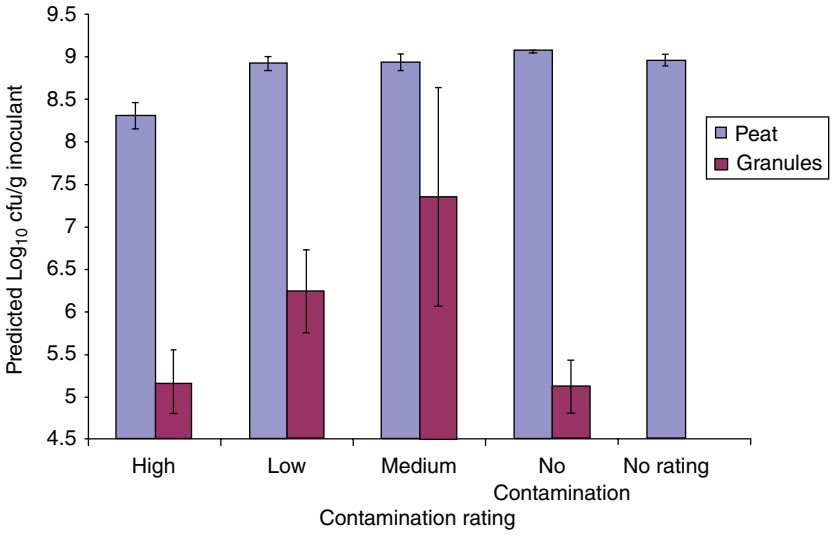

Figure 97.2 Number of rhizobia per gram of peat and granular inoculants at the point of sale as a function of contamination rating. Data were provided by ALIRU and counts of all inoculants were pooled and analyzed in GenStat using a general analysis of variance with unbalanced treatment design, with contamination rating as a factor. There was a significant effect of contamination rating for peat inoculants only [$P < 0.001$] with high levels of contamination resulting in a significant decrease in rhizobial numbers. There was no significant effect of contamination rating on rhizobial numbers in granular inoculants.

(Roughley et al., 1995). Detection of contamination at the point of sale is not generally a cause for action. However, removing the restriction on contamination in moist peat detected at the point of manufacture should not be considered and should therefore continue to be an important criterion for determining inoculant quality of freshly manufactured and re-submitted peat batches.

In a survey of 40 commercial North American rhizobial inoculants, Olsen et al. (1994b) found that in all but one of the inoculants tested contaminant organisms occurred in higher numbers than rhizobia (see Chapter 101) and they listed the potential negative effects of contaminants in peat. Contaminants may be pathogenic to humans, animals, or plants. They may be antagonistic to rhizobia, inhibit legume root nodulation, decrease the potential shelf life of inoculants, and increase the difficulty in performing quality control procedures. Brockwell (1982) suggested that low dilution "skips" in plant infection MPN tests may be due to inhibition of nodulation caused by other organisms such as saprophytic fungi.

The survey data for granular inoculants, while not extensive, do not indicate an interaction between level of contamination and numbers of rhizobia. This is probably due to the lower moisture content of granular inoculants and hence little likelihood that growth inhibition would occur during storage. Moisture status is clearly a major factor determining the potential effect of the level of contaminants within inoculants. In recent research, we found that moist preinoculated lucerne seed [Aw 0.84] had a much higher frequency of contamination (83.3%) than dry seed (38.9%, Aw 0.62) indicating that an increase in growth of contaminants will occur in ideal conditions (Deaker et al., 2012). Consequently, rhizobial survival was better on drier seeds after long-term storage.

While not all contaminants behave the same, it is impossible to determine which contaminants might be present in a carrier given the heterogeneous nature of the natural materials on which they are based. To maintain quality, it is recommended that restrictions on contaminants in moist peat inoculants remain and that contaminants in drier granular products be tolerated provided that rhizobial numbers remain above minimum standards. However, further research is necessary to determine whether high numbers of contaminant organisms in dry granules pose a threat to successful nodulation or affects the predictability of shelf life of the inoculant (see Chapter 101).

97.8 INOCULUM POTENTIAL AND INOCULANT EFFICACY

The minimum standards for rhizobia required for adequate nodulation of legumes have generally been presented as numbers per seed and vary depending on seed size. Globally, there is general agreement that these numbers should be 500 cfu/seed for legumes with very small seeds, 10^3 cfu/seed for small seeds, 10^4 cfu/seed for medium seeds and 10^5 cfu/seed for large seeds (Lupwayi et al., 2000, Herridge et al., 2002). Small seeds, in this case, refer to seeds of the size of subterranean clover [referred to as medium seeds in some texts] and very small seeds refer to seeds the size of white clover.

There is evidence that increasing numbers above these levels can greatly increase nodulation, nitrogen fixation and yield in both competitive (Ireland and Vincent, 1968) and non-competitive soil environments (Hume and Blair 1992; Roughley et al., 1993). Olsen et al. (1994a) suggested that minimum standards for rhizobial number for seeds should be increased 10-fold to 10^4, 10^5, and 10^6 per seed for each seed size category. However, in recent field experiments where faba bean was inoculated with increasing rates of a freeze-dried inoculant, Denton et al. (2013) did not find a yield benefit in competitive soil unless rates were increased 100 fold, which is uneconomical and impractical for peat and granular technologies. However, clear responses to increasing rhizobial numbers were observed in soil with no history of faba bean. Thompson (1991) noted that legume yield is not decreased when rhizobia are applied in excess and therefore there is no conceivable maximum standard for rhizobial inoculants.

We calculated the theoretical inoculum potential for different legumes using minimum standards for peat inoculants at manufacture (data not shown, 10 fold higher than at expiry) and at expiry and using recommended rates of inoculation (Table 97.3). We also calculated the theoretical inoculum potential in soil using the minimum standards for rhizobial number per seed and maximum and minimum published sowing rates. In order to calculate the number applied to seed, the number of seeds per kilogram for each species were averaged across a range of published values and cultivars ranging in size. When the colony-forming units per gram peat at expiry [1×10^8] was used to calculate colony-forming units per seed, only the number on faba bean exceeded the minimum standards for the large-seeded grain legumes. The lowest number was on narrow leaf lupin seed at 2.5 times lower than the standard. Standards were reached for all medium- and small-seeded species. This may suggest a need to increase the minimum standard at expiry or the rate of application of inoculant to seed.

The difference in numbers of rhizobia per seed on faba bean and other grain legumes is diminished when considering the number per hectare delivered at different sowing rates. Except in the case of soybean, the number of rhizobia delivered on large grain legume seed per hectare with the minimum standard of 1×10^5/seed and at the lowest sowing rate, was more than 1.5 times that of faba bean. The number of rhizobia delivered on narrow leaf lupin was more than 2.5 times the number delivered on faba bean. The low sowing rate for soybean of 15 kg ha^{-1} is used in some dryland

Table 97.3 Application of peat-based legume inoculants to seed; inoculum potential on seed and in soil

Legume species	Inoculum potential on seed at expiry (cfu/seed)	Minimum standard (cfu/seed)	Published sowing rates (kg/ha)		Inoculum potential in soil at different sowing rates (rhizobia/ha)	
			Lowest	Highest	Lowest	Highest
Grain legumes						
Faba bean	1.82×10^{5}	1×10^{5}	117	175	1.6×10^{10}	2.4×10^{10}
Chickpea	6.03×10^{4}	1×10^{5}	59	131	2.5×10^{10}	5.5×10^{10}
Pea/vetch	5.50×10^{4}	1×10^{5}	77	165	3.5×10^{10}	7.4×10^{10}
Lupin (angust.)	3.80×10^{4}	1×10^{5}	65	73	4.3×10^{10}	4.8×10^{10}
Lupin (albus)	8.32×10^{4}	1×10^{5}	153	197	4.6×10^{10}	5.9×10^{10}
Soybean	4.68×10^{4}	1×10^{5}	15	40	8.0×10^{9}	2.1×10^{10}
Mungbean	1.35×10^{4}	1×10^{4}	12	30	2.2×10^{10}	5.6×10^{10}
Lentil	2.51×10^{4}	1×10^{4}	50	75	9.9×10^{10}	1.5×10^{11}
Pasture legumes						
Sub. clover	3.89×10^{3}	1×10^{3}	4	10	5.2×10^{8}	1.3×10^{9}
Annual medic	2.24×10^{3}	1×10^{3}	3	8	6.8×10^{8}	1.8×10^{9}
Serradella	1.82×10^{3}	1×10^{3}	0.5	10	1.4×10^{8}	2.8×10^{9}
Lucerne	2.24×10^{3}	1×10^{3}	1	15	4.4×10^{8}	6.7×10^{9}
Red clover	1.86×10^{3}	1×10^{3}	1	4	5.4×10^{8}	2.1×10^{9}
Biserrula	2.88×10^{3}	1×10^{3}	1	3	8.6×10^{8}	2.6×10^{9}
Persian clover	6.76×10^{2}	5×10^{2}	4	10	5.9×10^{9}	1.5×10^{10}
Lotus pedunc.	1.62×10^{3}	5×10^{2}	1	3	1.5×10^{9}	4.6×10^{9}
White clover	6.31×10^{2}	5×10^{2}	0.5	4	8.0×10^{8}	6.4×10^{9}

Inoculum potential on seed is calculated using the recommended inoculant application rates and the number of seeds per kilogram calculated for each legume species as an average across several cultivars (Gemell, unpublished data). Inoculum potential in soil is calculated using minimum standards per seed.

cropping areas and it would be recommended that higher inoculant rates be applied when low sowing rates are used. Interestingly, only lentil at the high sowing rate delivered 10^{11} rhizobia per hectare when calculated using the minimum standards for seed inoculation. This number per hectare was selected as the minimum standard for granular inoculants to be comparable with the number delivered by seed. Although in most cases seed inoculation delivers almost an order of magnitude less rhizobia per hectare when calculated in this way, it is expected that numbers per hectare would be higher in reality as the majority of peat inoculants generally contain several times the minimum number of rhizobia at expiry and numbers per seed would exceed the global standard. However, when rhizobia are delivered on seed, rhizobial death rates can be high.

The question remains whether the numerical standards for legume inoculants are adequate to produce meaningful responses across a broad range of sites. Brockwell and Bottomley (1995) reported that most literature reports numbers of 1×10^{4} indigenous rhizobia/g soil, which is equivalent to 1.5×10^{13} rhizobia/ha if spread evenly through the top 10 cm soil. It is clearly inconceivable that the inoculant strain applied at the standard recommended rate could outnumber indigenous rhizobia. The authors suggest that numbers of indigenous rhizobia between 10 and 1000 rhizobia/g soil are of much more practical concern, particularly if the soil population is poorly effective. In this case, the inoculant strain should be applied in high numbers or be highly competitive; however, seed inoculation may improve competitiveness by localizing inoculant strains around the emerging root.

The number of rhizobia delivered per hectare on pasture legume seed is generally one to two orders of magnitude lower than numbers from grain legumes because of much lower sowing rates. In this case, it would be important to localize rhizobia around the emerging root using seed inoculation, and the number per seed is critical for improved competition by the inoculant strain. Roughley and Walker (1973) recommended farm inoculation of pasture seed where sowing rates were low. The number per hectare is more relevant when inoculants are delivered directly to the soil.

97.9 SOIL VERSUS SEED INOCULATION

The prospect of inoculating legumes by direct application of inoculant to the soil has been investigated for some time. Brockwell et al. (1980) compared application of solid and liquid inoculants directly to the soil with slurry inoculation on seed. The inoculants were evaluated in 16 field experiments over 8 years with soybean, chickpea, sweet lupin, field

pea, and subterranean clover. They listed the inefficiencies associated with seed inoculation as follows:

1. Seeds may be treated with incompatible chemicals for the control of fungal disease, etc.
2. High sowing rates of grain legumes render seed inoculation a major and difficult task.
3. Seeds may be too fragile to be tumbled with the inoculants; such is the case with peanuts.
4. Some legumes lift their seed coats, and hence inoculant rhizobia, out of the soil during emergence.
5. The size of the seeds (particularly small seeds) restricts the number of rhizobia that can be applied.
6. Inoculation of some seeds can mobilize toxic water soluble compounds from the seed coat.

The authors found that under favorable conditions solid and liquid inoculants applied directly to the soil were generally as good as seed inoculation (as measured by nodule occupancy, quality of nodulation, seedling establishment, shoot dry matter production, and seed yield). Soil inoculants outperformed seed-applied inoculants when conditions on the seed were unfavorable for survival (e.g., use of seed-applied chemicals). Brockwell et al. (1980) concluded that while granular inoculants have advantages, their use would require modification of seeding equipment and adequate storage space, and transport costs would be increased because of the need for large quantities.

Several other authors have reported superior performance of soil-applied inoculants with soybean (Chamber, 1983; Hardarson et al., 1989; Gault et al., 1994), field pea (Jensen, 1987; Rice et al., 2000; Kutcher et al., 2002; Clayton et al., 2004), chickpea (Kyei-Boahen et al., 2002; Gan et al., 2005) and to a lesser extend with alfalfa (Knight, 2007). Granular inoculants were particularly advantageous when seed-applied fungicides were used (Kutcher et al., 2002) or where seeds were sown into acid soil (Rice et al., 2000). In most studies, granular inoculants and seed-applied peat slurries outperformed seed-applied liquid inoculants.

Results from field studies over several years on the use of granular inoculants for inoculation of field pea, faba bean, lentil, chickpea, and narrow leaf lupin in Victoria, New South Wales, and South Australia were compiled by Denton et al. (2009). In all experiments, seed inoculation with peat slurries were the same if not better than granular inoculation. The results suggest a strong interaction between rhizobial numbers in granules and nodulation (percentage of plants nodulated). Nodulation percentage increased markedly when rhizobial numbers per gram of inoculant increased from 10^6 to 10^7 (equivalent to 10^{10} to 10^{11} rhizobia/ha when applied at $10\,kg\,ha^{-1}$). The authors concluded that the success of inoculation was also related to particle size distribution of the granules. It is interesting to note that attapulgite granules containing 10^6 rhizobia/g but with a predominantly smaller particle size (highest frequency at 0.5–1.0 mm) were as effective as larger granules with 10 times more rhizobia per gram. Granules with low numbers and larger particle sizes were the least effective. Attapulgite granules were also more effective than peat slurry inoculation when applied to soybean [ALIRU annual report, 2005]. It follows that particle size limits the distribution of granules and hence rhizobia, and thus should be a determinant of inoculant quality.

Inoculant manufacturers recommend application rates for granular inoculants according to row spacing. It is expected that row spacing is the most critical factor as this would affect granular distribution by essentially varying the number of linear meters for application. Increased nodulation would also potentially result from higher plant densities by increasing the probability that granules would be proximal to roots.

Plant density for legume crops (as affected by sowing rate and row spacing) varies for a number of reasons including rainfall, temperature, cultivar and seed yield potential, and disease (Regan et al., 2003). Row spacing of grain legumes may be as wide as 100 cm [NSW DPI Summer Crop Production Guide, 2008]. Wide spacing is an advantage when sowing into stubble or in moisture-limited situations. In this situation, there would be 10,000 linear meters per hectare and granules applied at $10\,kg\,ha^{-1}$ would be distributed at $1\,g\,m^{-1}$. A decrease in row spacing would effectively increase the number of linear meters for application and decrease the number of granules per meter if the same application rate was used. If granule size was large, then there would be considerably less confidence in distribution of granules when narrow row spacing was used. It is suggested that consideration be given to developing a standard for granule size and the minimum number of granules (as well as a minimum number of rhizobia) per linear meter applicable to the lowest plant density.

Clearly, soil applied inoculants can be effective and overcome constraints associated with seed inoculation as well as providing a convenient ready-to-use product. However, their efficacy is dependent on numerical quality and issues relating to supply and application of large quantities of inoculant. While liquid inoculants applied to seed did not have much success in the described studies, they provided a convenient alternative to peat for seed inoculation (Hynes et al., 1995).

Freeze-dried and liquid inoculants applied to seeds are exposed to similar potentially adverse conditions as peat slurries. However, there is evidence of physiological changes in peat improving tolerance (Feng et al., 2002; Casteriano et al., 2013). Conversely, cells in liquid cultures became more membrane-compromised with age (Maurice et al., 2001). Peat also provides additional protection to cells on seed (R. A. Date, unpub. PhD thesis, University of Sydney, 1959). Survival is strain dependent and can be improved by selection of a protective polymer (Deaker

et al., 2007; Deaker et al., 2012; Hartley et al., 2012). McLeod and Roughley (1961) reported the successful use of freeze-dried inoculants in field trials; however, survival on seed after application with polymeric adhesives was poor (Vincent, 1965). Freeze-dried rhizobia generally survive better at high temperatures than peat cultures but the cells must be kept dry to maintain tolerance to high temperatures (Kremer and Peterson, 1982, 1983). Studies on exposure of freeze-dried cultures to high temperatures on seed revealed that survival was poor when relative humidity was increased (McInnes and Date, 2005). However, the ability to apply large quantities of freeze-dried inoculant to seed and increasing rhizobial numbers by orders of magnitude may be an advantage (Denton et al., 2013) but is not currently economically viable.

97.10 QUALITY OF COMMERCIALLY INOCULATED [PREINOCULATED] LEGUME SEED

Surveys of preinoculated seed in Australia between 1972 and 1974 revealed that five (three batches of subterranean clover and two batches of lucerne) of the 48 batches of seed tested had more than 1600 rhizobia/seed (Brockwell et al., 1975). Further six batches had between 640 and 1600 rhizobia/seed, one of which was white clover and the rest were subterranean clover. Therefore, a total of 23% of the batches tested passed or came close to passing current AIRG standards. In the period 1999–2003, of 293 samples tested the results were more promising for lucerne with 73% passing standards (Gemell et al., 2005). However, only 32% of subterranean clover, 3% of white clover, 4% of red clover, and 0% of other species passed the standards. More recently, 287 samples of preinoculated seed were collected and tested between 2005 and 2010 and only 10% of samples passed the AIRG standards (O'Hara et al., 2012).

Our recent research indicates that this decrease in quality is related to changes in the commercial inoculation process, most notably the poor compatibility of selected materials that are incorporated into the seed pellet and the drying rate of inoculated seeds (Deaker et al., 2012; Hartley et al., 2012). It is our understanding that coating materials are generally selected on the basis of processing quality and the physical properties of the final product rather than on compatibility with rhizobia.

Major changes to production are required to improve preinoculated seed quality, and challenges relating to rhizobial survival on seed, in general, need to be addressed if this product is to reach its full potential. Large areas of grazing pasture and rangelands rely almost entirely on inputs of nitrogen fixed by legumes. It is likely that nitrogen fixation is suboptimal in these environments given the now widespread use of an essentially ineffective inoculation technique. In this case, nodulation and nitrogen fixation are dependent of the effectiveness of the resident soil rhizobia population, which may be substantially less effective than selected inoculant strains as demonstrated by Drew et al. (2012).

97.11 CONCLUSIONS

We conclude that true benefits of inoculation of legumes will result from inoculants that are not only of high quality but are also easy to apply in a way that promotes both adoption of the practice and survival of effective rhizobia. Standards should be reviewed for each formulation and modified accordingly so that inoculant properties support the efficacy of application (see Chapter 101).

The efficacy of seed inoculation is dependent on the initial number and physiology of rhizobia in the inoculant, rhizobial strain, method of application including coating procedure and compatibility of polymer adhesives used during seed coating, and length of time on seed before sowing. If these conditions are optimized, placement of rhizobia proximal to the emerging root for prompt colonization and nodulation and the requirement for smaller quantities of inoculant would be advantageous. Clearly, a major development is required to improve the quality of preinoculated pasture legume seed; however, the convenience of this product has resulted in its widespread use. Given the largely unknown but potentially suboptimal effectiveness of resident soil rhizobia, large gains in nitrogen fixation could be made in the vast areas of pasture and rangelands by improving the quality of preinoculated seed.

In the absence of ideal conditions or where there is adequate means, soil inoculation providing adequate distribution of rhizobia could be recommended. However, there is little data on inoculant quality of granular products either at the point of manufacture or in the retail market, and the shelf life has not been confirmed. In addition, the need for high volumes and increased input costs may reduce the adoption of this product.

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Chapter 98

Rhizobiophages as a Marker in the Selection of Symbiotically Efficient Rhizobia for Legumes

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98.1 INTRODUCTION

The traditional approach for the development of inoculants has consisted of isolation, testing, and selection of a single strain with the desired properties, such as high nitrogen fixation ability in symbiosis with selected host plants. In cases where indigenous rhizobia able to nodulate that host are already present in the soil but are less efficient, the capacity of the inoculant strain to outcompete those rhizobia is important. The selection of efficient strains is still hampered by the fact that, although the genes encoding nodulation and nitrogen fixation in rhizobia are known (e.g., Franche et al., 2009), the precise genetic bases for symbiotic effectiveness, competitiveness, and tolerance to environmental stress factors in soils remain largely unknown.

Various biotic and abiotic factors are known to influence the survival and competitiveness of rhizobia in soils (see Chapter 97). Bacteriophages are one of the potent factors that can affect the outcome of *Rhizobium*–legume symbiosis (Kowalski et al., 2004). Rhizobiophages have been isolated from soils of many countries and they are usually associated with legume cultivation (Amarger, 2001). Identification and selection of efficient *Rhizobium* strains from field-grown soils is a difficult task; there is a need for a dependable screening system of sufficient sensitivity such that each individual may be easily differentiated from other strains of diverse origins.

Phages associated with susceptible rhizobial strains have been isolated from rhizospheric soil and used to characterize indigenous rhizobial population (Schmidt et al., 1986; Appunu and Dhar, 2006a). The occurrence and distribution of phages active against various groups of rhizobia have been reported from India (Dhar et al., 1979; 1980; Singh et al., 1980; Dhar and Ramkrishna, 1987; Dhar et al., 1993), Poland (Staniewski, 1970), New Zealand (Patel, 1976), Canada (Lesley, 1982), and Senegal (De Lajudie and Bogusz, 1984). Recently phages infective on soybean and lentil rhizobia have also been isolated and characterized (Appunu and Dhar, 2008; Jaiswal and Dhar, 2010).

Rhizobia have long been known to differ in phage susceptibility, and recently this feature has been used in diversity studies of rhizobial strains from field soils (Bromfield et al., 2010). Phages could play an important role in the development of ecologically competitive rhizobial strains in soil (Kowalski et al., 2004; Bromfield et al., 2010).

Variation in host specificity of phages has been suggested to be due to the diversity of the host bacterium (Holmberg et al., 1984). The bacterial wall is likely to be significant in determining the host specificity of phages (Defives et al., 1996). Outer membrane-bound lipopolysaccharides (LPSs) and proteins serve as receptors for phages (Werts et al., 1994). Defives et al. (1996) and Sharma et al. (2008) reported that the involvement of the O-antigen of LPS of bacteria help attract phages. So far, there are no publications on the possible biochemical or structural

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factors underlying the recognition between specific phages and certain highly efficient rhizobial strains. It is assumed that high susceptibility of rhizobia that show the most symbiotic effectiveness with their host to phages may involve repeating units of the O-antigen of lipopolysaccharide. Therefore, phage specificity in conjunction with micro-heterogeneity in LPS profiles (LPS subtypes) can be used to easily discriminate rhizobia within the same species.

98.2 PHAGE TYPING

Phage sensitivity pattern has been suggested as a potent method to differentiate bacteria according to their genotypic and phenotypic traits (Thurman and Bromfield, 1988; Lindstrom and Lehtomaki, 1988; Sharma et al., 2002). In addition, it gives fast and reproducible results to discriminate indigenous rhizobial isolates and proves to be useful enough for monitoring the purity of bacterial germplasm (Lesley, 1982; Lindstrom et al., 1983; Appunu and Dhar, 2006b). Phage typing has been widely used to characterize strains of *Rhizobium trifolii*, *Rhizobium leguminosarum* for pea, vetch, and horse bean, *R. meliloti* strains and one strain of *R. lupini* for lupine (Staniewski, 1970), *Rhizobium meliloti* of alfalfa (Lesley, 1982), *R. leguminosarum* of *Galega oficinalis* (Lindström et al., 1983), *Bradyrhizobium* sps. of soybean (Appunu and Dhar, 2006b), and *R. leguminosarum* bv. *viciae* of lentil (Jaiswal et al., 2012a). Phage typing simplifies the problem of identification of inoculation strains in soil (Lindstrom and Lehtomaki, 1988; Lindstrom et al., 1990; Anand et al., 2012).

Rhizobiophages are very specific to their host strains and have been reported as an authentic genetic marker for differentiating rhizobial strains. This has been used to differentiate the rhizobial strains of chickpea (Dhar and Ramkrishna, 1987), pigeonpea (Dhar and Kumar, 1998), frenchbean (Dhar et al., 1993), cowpea (Singh et al., 1980), soybean (Hashem et al., 1996; Ali et al., 1998; Appunu and Dhar, 2008), pea (Mendum et al., 2001), lentil (Jaiswal and Dhar, 2010), and some other legumes (Novikova et al., 1993). Rhizobiophages could also play an important role in the development of ecologically competitive phage-resistant strains (Bromfield et al., 2010; Kowalski et al., 2004). Phage could have a fundamental role as an indicator of the microdiversity that is required to efficiently exploit ecological resources (Suttle, 2007).

98.3 MATERIALS AND METHODS

The phage typing procedure as described by Lesley (1982) was used invariably to test the host range sensitivity pattern of a bacterial strain. Phage strains specific to their host rhizobia in terms of their lytic activity were utilized for phage typing of indigenous rhizobial isolates. Each rhizobial strain in the stationary growth phase was introduced into soft agar (0.7%) medium and plated by the double agar method. A volume of 50 µl of each phage strain (10^8 pfu ml^{-1}) was spotted on the bacterial lawn shortly after the soft agar layer solidified. Plates were examined for lytic activity on the lawn following 4–5 days of incubation (Fig. 98.1).

Four rhizobial isolates are shown to have different lytic reactions with six phage strains. Rhizobial strain MPSR084 was found susceptible to three phage strains (RT1, RT3, and RT5) while strain UPSR020 showed a clear lytic zone with two phage strains (RT2 and RT4). Isolates MPSR033 and MPSR220 revealed lytic zones only with phage strains RT5 and RT6, respectively (Fig. 98.1).

98.4 RESULTS AND DISCUSSION

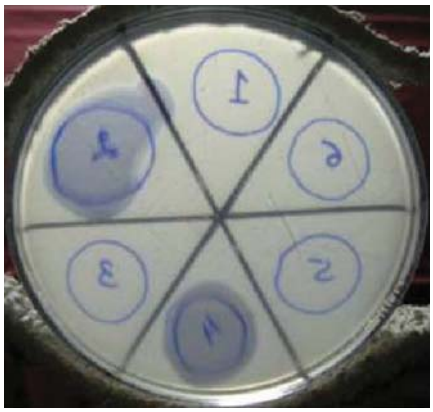
On the basis of sensitivity patterns of lentil (*Lens culinaris*) rhizobial strains, 10 phage groups were identified and the number of rhizobial isolates belonging to each phage typed group is given in Table 98.1. The phage strains used showed variation among the susceptible strains for a particular group. Phage group I included three phage-sensitive strains while group VI contained three susceptible strains to phage LRP-20 only. The maximum number of isolates belonging to phage groups VII and VIII were lysed by two phages LRP-4 and LRP-15. Phage isolates were able to infect the rhizobial strains of *Pisum sativum* and *Phaseolous vulgaris* (Jaiswal et al., 2012a).

Although some rhizobiophages have shown a narrow host range (Dhar et al., 1978; Hashem et al., 1986), many have exhibited a wide host range (Barnet, 1972; Dhar and Ramkrishna, 1987). Host range is, however, not an invariant characteristic of a phage strain and can be altered, as bacteriophages can adapt themselves to proliferate on different host cells.

98.5 SYMBIOTIC RESPONSE OF PHAGE-TYPED *Rhizobium* WITH HOST PLANT

The presence of phage in soil may cause selection pressure on sensitive rhizobial strains, and thus their continued coexistence with the sensitive host genotype is important (Hashem and Angle, 1988; Kleczkowaska, 1971; Patel, 1976). Under such selection pressure, a phage-resistant form of *Rhizobium* may create a better fitness in the rhizosphere. The knowledge of associated changes in the symbiotic properties of such variants is important. Symbiotic effectiveness of *Rhizobium* strains belonging to different phage-sensitive and phage-resistant groups was compared on host plants grown in a nitrogen-free culture system. A significant difference in nodule number, ARA, and total dry matter accumulation was observed. Most of the phage-marked

Isolate MP SR084 showing lytic zone with three phage strains



Isolate UP SR020 showing lytic zone with two phage strains

Isolate MP SR033 showing lytic zone with single phage strain

Isolate MP SR220 showing lytic zone with single phage strain

Figure 98.1 Plates seeded with indigenous bradyrhizobial isolates showing lytic reaction with the tested phage strains.

Table 98.1 Phage-typing pattern of indigenous *Rhizobium leguminosarum* isolates and their distribution

Phage Group	Pattern of lysis (+) with Phage Strains								Total Number Belongs	Sensitive Number Distributed to the Rhizobial Isolates from		
	LRP-1	LRP-4	LRP-13	LRP-15	LRP-16	LRP-20	LRP-21	LRP-24		*Lens culinaris*	*Pisum sativum*	*Phaseolus vulgaris*
I	+	+		+					14	10	3	1
II		+					+		7	7	0	0
III				+				+	8	7	1	0
IV	+			+					12	2	10	0
V			+				+		4	4	0	0
VI						+			3	3	0	0
VII		+		+					21	9	8	4
VIII		+					+		19	15	1	3
IX				+				+	9	4	0	5
X					+	+			8	8	0	0
Total									**105**	**69**	**23**	**13**

strains displayed significantly higher symbiotic parameters than phage-resistant strains. In lentil (*Lens culinaris*), reported by Jaiswal and Dhar (2011), four phage susceptible strains (BHULR-104, BHULR-113, BHULR-115, and USDA 2431) were found significantly superior than others in terms of symbiotic parameters. The isolate BHULR-115 exhibited the highest nodule number and plant dry weight, whereas the isolate BHULR-113 evidenced superior performance with regard to nodule number, ARA, and plant dry matter production. Overall increase in plant dry weight by phage-susceptible strains was about 12% more than by all phage-resistant strains.

These genetically distant groups may differ in their ability to persist in soil, infect the host cell, or fix nitrogen in the nodule (Appunu and Dhar, 2006c; Meghvanshi et al., 2005; Jaiswal and Dhar, 2011; Jaiswal et al., 2012b). Phage typing has been also used to identify the successful competitor when alfalfa (Lesley, 1982) and soybean (Anand et al., 2012) plant roots were inoculated with phage marked rhizobial strains under axenic and field conditions.

Phages that are used for typing of rhizobia differ in plaque shape, for example, possessing either circular, sharp, or hazy margins (Dhar et al., 1978; 1979; 1980; 1993; Dhar and Kumar, 1998; Sharma et al., 2002; Appunu and Dhar, 2006c; Jaiswal and Dhar, 2010). Plaque type diversity is mainly associated with the host but habitat factors also influence plaque type groups for at least soybean native *Rhizobium* isolates. Perhaps these phages could be used as an indicator for the distribution of a particular type of rhizobia present in the soil. Phages were also used successfully in the characterization of indigenous rhizobia residing in soybean nodules (Schmidt et al., 1986; Thurman and Bromfield, 1988; Ali et al., 1998; Appunu and Dhar, 2006a).

Ahmad and Morgan (1994) reported that when the concentration of phage is increased in comparison to rhizobia in soil, cowpea nodulation is decreased. Hashem and Angle (1988) have also shown that phages of *B. japonicum* cause a significant reduction in nodule number, nodule mass, and shoot dry weight of soybean. Staniewski et al. (1962) suggested that the direct lytic action of the phage on the nodule bacteria depends on the bacteriophage population in soil. And this finding opened the way to select phage-resistant rhizobial strains.

Recently, it has been observed that the phage-resistant soybean bradyrhizobial isolates form nodules and fix nitrogen on a broad host range of legumes (*Vigna mungo, Vigna radiate, Vigna unguiculata and Cajanus cajan*) (Jaiswal et al. 2012b; Appunu et al., 2009).

98.6 CONCLUSION

Phage typing results indicate the great utility of this method to differentiate nodule rhizobial isolates at a strain level. Compared to serological analysis, phage typing is a simple and less time-consuming technique to differentiate the bacterial strains if a number of lytic phages are available. It provides a basis for selection of rhizobia, which may have strategies for application of appropriate inocula to improve legume production and provide information of genetic diversity and functional variability of the legume–*Rhizobium* symbiosis.

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Chapter 99

Nitrogen Fixation with Soybean: The Perfect Symbiosis?

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99.1 INTRODUCTION

Soybean [*Glycine max* [L.] Merr.] is the most important legume cropped worldwide. The high protein content of its grains – about 40% – represents a nutritionally important part of the diets of humans and domestic animals. In addition, other seed products have broad industrial and pharmaceutical applications. For example, there is increasing interest in using soybean oil for biodiesel production to replace its fossil-based counterpart.

In 2011, the total area cropped to soybean was estimated at 103 million ha, with a total production of 261 million metric tons (FAO, 2011). The three major producers are the United States, Brazil, and Argentina (Fig. 99.1); South American countries account for 52% of global production. Interestingly, increasing production has been reported in Africa – currently cropping 1.5 million ha – and 12.1 million ha are cropped in "low-income food-deficit countries" (FAO, 2011), indicating that the legume may play an increasingly key role as a protein source for impoverished populations. The increased production in these areas can be attributed to relatively low susceptibility to pests and diseases, better grain-storage quality, high leaf-biomass yield (benefiting soil fertility], and secure commercial markets (Mafangoya et al., 2009).

Despite considerable differences in yield among countries, for example, averages of 2789 and 2862 kg ha^{-1} in North and South America, respectively, and of 1186 and 1220 kg ha^{-1} in Africa and in countries classified as "low-income food-deficit countries," respectively (FAO, 2011), one may conclude that reasonable yields may be achieved even with low-input management practices. Considering the high demand of N by soybean crops, of about 80 kg of N per 1000 kg of grain (on average 65 kg N in the grain and 15 kg N in the vegetative crop residues, including the roots) (Hungria et al., 2007; 2009), and that the great majority of the soybean in South America, Africa, and in some Asian countries is cropped on soils of very low N content and with no application of N-fertilizer, we may conclude that, in a variety of environments, biological nitrogen fixation (BNF] makes significant contributions to soybean crops.

In an exercise to quantify the global contribution of soybean BNF, Herridge et al. (2008) came up with a figure of 16.4 Tg annually, representing 77% of the N fixed by all crop legumes. However, the increasing shift in production from the United States – with high inputs of N-fertilizers to the soybean-maize cropping system – to the low N-fertilizer input agriculture practiced in South America and Africa should soon result in significantly greater contributions from BNF.

In a survey of quantifications of BNF by agricultural legumes, soybean was rated as the highest among the grain producers, with contributions of up to 450 kg N ha^{-1}, surpassed only by the fodder crops bitter lupin (*Lupinus mutabilis* Sweet) and alfalfa (*Medicago sativa* L.), with up to 527 and 470 kg N ha^{-1}, respectively (Ormeño-Orrillo et al., 2013). On the other hand, few reports exist of low contributions of BNF by soybean; there is need for research

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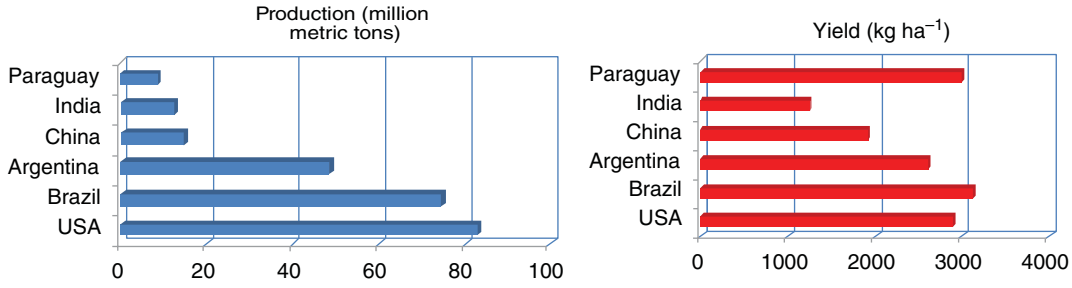

Figure 99.1 Soybean production and yield in the six main producers globally, 2011 (after FAO, 2011).

on limiting factors related to such cases. We hypothesize that the majority of reports of low BNF contributions result from breeding and cropping managements with high inputs of N-fertilizers. Attention should be paid also to global environmental changes, with emphasis on increasing periods of drought that may represent serious limitations to BNF, highlighting the need to identify genotypes with high BNF and high tolerance of moisture deficiency (Chen et al., 2007; Sinclair et al., 2007; Purcell, 2009). Other factors may be critical to the success of the symbiosis, such as treatment of seeds with pesticides (e.g., Campo et al., 2009; Hungria et al., 2009).

99.1.1 Soybean Domestication, Cropping, and Breeding for Nitrogen Fixation

Domesticated soybean emerged in central and northern China in the period 1100–700 B.C., moving before the first millennium A.D. to India, Nepal, Burma, Thailand, Indochina, Korea, Japan, Malaysia, Indonesia, and the Philippines (Hymowitz and Singh, 1987). It arrived in Europe (Germany) in 1712, in Paris in 1740, and in the United States in 1804, probably from seed exchanges with France (reviewed in Hungria et al., 2005). Significant production in the United States and Europe did not occur until the beginning of the twentieth century (Hymowitz, 1970), increasing with the collection and maintenance of germplasms. In South America, *Glycine* spp. was introduced – mainly as seeds from the United States – in 1880 to Argentina, in 1882 to Brazil, and in the 1920s to Paraguay (Hungria et al., 2006a). However, despite the hundreds of accessions available in the main producing countries, it is noteworthy that the genetic base used in occidental breeding programs is narrow. For example, Delannay et al. (1983) reported that, in the United States, 10 introductions provide 80% of the gene pool; similarly for the Brazilian germplasm (Hiromoto and Vello, 1986).

Another limitation is that although N_2-fixation rates of 1–2 kg N ha^{-1} per day should be possible in all legumes, with an emphasis on the efficient symbiosis with soybean (Giller, 2001), the low cost of N-fertilizers in developed countries such as the United States has led to dependence on fertilizers for N, with corresponding reductions in the contributions of BNF to soybean (van Kessel and Hartley, 2000). This helps explain the low interest in breeding for increased BNF in US breeding programs (see Chapter 106). To attend to the demands of high-yield cultivars in the presence of mineral N, supernodulating soybean mutants, tolerant of nitrate (nitrate tolerant symbiotic, *nts*), were developed (Carroll et al., 1985; Gremaud and Harper, 1989; Akao and Kouchi, 1992; see Chapter 106). However, field evaluations of *nts* mutants usually provided lower yields (Wu and Harper, 1991) and some mutants with excessive nodulation show increased sensitivity to environmental stresses. It was suggested that the nodules on *nts* mutants consume extra carbohydrate and, in occupying more root surface, compromise absorption of mineral N (Takahashi et al., 1995).

An early breeding approach in the United States toward increasing the contribution of BNF was based on the identification of soybean genotypes that restrict nodulation by dominant indigenous serogroups of *Bradyrhizobium* that fix N_2 with low efficiency. Some of the genes identified were rj_1, Rj_2, Rj_3, Rj_4, rj_5, rj_6, rj_7, and Rf_{g1}, all distinct in their segregation, which facilitates breeding (Devine, 1984; Sadowsky and Cregan, 1992; Hayashi et al., 2012). In a similar approach, Kvien et al. (1981) and Weiser et al. (1990) sought cultivars with various levels of preference for, or exclusion of, certain serogroups. Using the exclusion approach, Cregan and Keyser (1986) identified, among plant introductions (PIs) and modern genotypes, some with restricted nodulation to serogroup 123, and promising results were obtained with the Rj_4 genotypes (Sadowsky and Cregan, 1992). However, although the presence of indigenous bradyrhizobia of serogroup 123 has been considered a main limiting factor to BNF in midwestern United States (Kvien et al., 1981; Cregan et al., 1989; Weber et al., 1989), breeding programs to restrict nodulation by serogroup 123 strains have been discontinued.

A contrasting approach was taken in Africa – mainly Nigeria – in the 1980s, with the exploitation of cultivars with promiscuous nodulation with indigenous strains (Kueneman et al., 1984; Pulver et al., 1982). The approach was based on the difficulties of producing and applying inoculants in sub-Saharan countries. However, with commercial crops

being increasingly established in several African countries, difficulties in producing and importing inoculants have decreased, and, with the need to grow high yielding cultivars, there has been a change from promiscuous cultivars to those responsive to commercial inoculants. In addition, there are now reports that inoculation can increase the yields of promiscuous cultivars. Important initiatives are in progress, such as N2Africa project (http://www.n2africa.org).

With the exception of the United States, the high cost of N-fertilizers throughout the American continent has necessitated a reliance on BNF. Interestingly, most of the Brazilian genotypes are derived from North America, but the main achievement of the Brazilian soybean program has been that it has always relied exclusively on inoculation, commonly in soils that are limited in N (Hungria et al., 2005; 2006a; 2007; Vieira et al., 2010). However, even under environmental conditions favoring BNF, there are reports of differences among Brazilian soybean cultivars in their symbiotic performances (Hungria and Bohrer, 2000; Hungria et al., 2006a), highlighting the opportunity for improvements in rates of BNF, as well as the need to monitor the BNF capacity of newly released cultivars across environments.

Worthy of consideration are possible adverse effects on BNF of transgenes in genetically modified (GM] soybean cultivars. Commercial use of GM soybean – roundup ready [RR] glyphosate-tolerant lines – was initiated in 1996 in the United States. Soybean now represents the main GM crop, with 75.4 million ha grown worldwide in 2011 (ISAAA, 2011). Greenhouse and field studies evaluating the effect of glyphosate on the *B. japonicum*/*B. elkanii* symbiosis with RR soybean cultivars have shown deleterious effects on nodulation and/or BNF (e.g., Reddy and Zablotowicz, 2003; Zablotowicz and Reddy, 2004; Bohm et al., 2009). Under field conditions, decreases in nodulation, but not in yield, have been demonstrated (Hungria et al., 2014). In general, effects of transgenic soybean changed with glyphosate rate, salt and time of application, soybean variety, and geographical and environmental conditions, and could not be attributed to the transgene. Other generations of GM soybeans are now available, and a recent meta-analysis has shown that they perform better than their conventional counterparts in agronomic and economic terms (Areal et al., 2013). It should be borne in mind that weeds can compete for nutrients, water, and radiation, and insects can severely damage plants; therefore, without proper management of weeds and insects, contributions from BNF can be compromised (Fig. 99.2).

Finally, advances in our knowledge of plant genes related to BNF have been achieved with the help of molecular tools. A first report of a gene associated with nodulation resulted from cloning in *Lotus japonicus*; *nin-1* (a non-nodulating mutant) encoded a transcription factor related to the regulation of gene expression during root infection (Schauser et al., 1999; see Chapter 59). Since then,

(a) (b)

Figure 99.2 Field experiment to evaluate biological nitrogen fixation in (a) transgenic RR soybean managed with glyphosate, and (b) the nearly isogenic parental non-transgenic genotype managed with conventional herbicides. Experiment performed at Embrapa Cerrados, Planaltina, Brazil.

impressive progress has been achieved, with the identification of several genes involved in the root–nodule symbiosis, mostly by using two model legumes, *Lotus japonicus* and *Medicago truncatula*, which have relatively small, simple genomes and are amenable to molecular transformation (Oldroyd et al., 2011; Reid et al., 2011). Outstanding results have also been obtained as a result of the publication of the integrated linkage map of the soybean genome (Cregan et al., 1999; Song et al., 2004); from this map, quantitative trait loci (QTLs) have been broadly used in soybean to identify features of interest, such as yield potential, disease resistance, and contents of protein and oil in seeds (SoyBase, 2012; see Chapter 106); unfortunately, very few groups are including BNF genomic information in this map (Nicolás et al., 2006; Santos et al., 2006; Santos et al., 2013). However, with the outstanding knowledge obtained with the first sequenced genome of soybean (Schumutz et al., 2010), the large EST databases and high density linkage maps have enabled the isolation of several genes, for example, the *Rj* genes, genes related to the perception of Nod factors, early and late nodulins, and the NARK gene (nodule autoregulation receptor kinase] (Reid et al., 2011; Hayashi et al., 2012). Transcription and proteomic profiles of nodulated and non-nodulated soybeans have also identified a variety of genes expressed in the formation and function of the symbiosis (e.g., Brechenmacher et al., 2008; Nguyen et al., 2012; Carvalho et al., 2013), pointing the way to novel studies to elucidate the roles of a number of interesting genes.

In conclusion, soybean breeding has shown spectacular results, for example, average yields in Brazil have increased from 1100 kg ha^{-1} in the 1960s to more than 3000 kg ha^{-1} today. Breeding has focused mainly on increased crop yield, resistance to diseases, and improved seed quality and nutritional traits, in addition to adaptation to edaphoclimatic conditions, such as shorter day length and water-stress tolerance. Transgenic approaches have achieved tolerance of herbicides and resistance to insect predation. Unfortunately, as mentioned above and pointed by Nicolás et al. (2006) and Hayashi et al. (2012), relatively little effort has been

expended in regard to BNF ability, despite the importance of the process to seed productivity and quality.

99.1.2 Selection and Use of Elite Rhizobial Strains

In China, a variety of described and putative species of the genera *Sinorhizobium* and *Bradyrhizobium* nodulating soybean have been identified (e.g., Keyser et al., 1982; Yang et al., 2006; Man et al., 2008), supporting the hypothesis that the original center of the legume is also the center of diversity of compatible rhizobia. However, the need for selecting strains with higher capacity of fixing nitrogen has long been recognized (Baldwin and Fred, 1929; Fred et al., 1932); in the late 1920s, Fred and colleagues commented that native soil strains were not always effective, requiring a screening process to increase productivity by inoculation (Fred et al., 1932).

The most important soybean breeding programs have been in the United States, Brazil, and Argentina, and as these countries do not represent centers of genetic domestication of the host plant, when their soils have not been previously cropped with the legume and in the absence of inoculants, populations of compatible rhizobia are small or absent (e.g., Vargas and Suhet, 1980; Elmore, 1984; Brutti et al., 1998; Ferreira and Hungria, 2002). Therefore, soybean cropped for the first time in soils of low N content show significant responses to inoculation with selected strains (McLoughlin et al., 1990; Hungria et al., 2006a), as exemplified in Figure 99.3.

Interestingly, only a few *Bradyrhizobium* strains have been used in the breeding programs in these countries, resulting in cultivars being highly specific in their strain needs.

One strain broadly used in commercial inoculants is *B. japonicum* USDA (United States Department of Agriculture) 110 (=TAL 102, =BCRC 13528, =JCM 10833, =3I1B110, =ACCC 15034), isolated in 1959 from a nodule on a soybean grown in Florida, USA (Keyser and Griffin, 1987). The strain has been used since then in several commercial inoculants in the United States, and in Europe, Asia, and Africa. It is noteworthy that this strain was recently reclassified and is the type strain of the new species *B. diazoefficiens* (Delamuta et al., 2013).

(a) (b)

Figure 99.3 Soybean grown in first-year areas and soils poor on N. Contrasting effect of green plants inoculated and fixing nitrogen and yellowish non-inoculated plants. Experiments performed in (a) Planaltina, central Brazil, in the 1980s (Photo: Dr. M. A. T. Vargas, Embrapa) and (b) Boa Vista, northern Brazil, in the 2010s (Photo: Dr. J. E. Zilli, Embrapa).

Another strain broadly used is the "improved" 532C (=532), selected by Professor David Hume in 1988 after screening several strains received from the Nitragin Company in the United States; since then, it has been used in inoculants in the United States and, from 1990 also in Canada (Shurtleff and Aoyagi, 2010). Inoculants carrying strain 532C have been exported, and it was probably used in commercial inoculants in Brazil from 1965 to 1966 and in 1976 and 1978 (Santos et al., 1999; Hungria et al, 2006a).

B. japonicum CB 1809 (=USDA 136, =3I1b136, =TAL 379, =SEMIA 585, serogroup 122) (now reclassified as *B. diazoefficiens*, Delamuta et al., 2013) was isolated in United States and sent to Australia, where it was characterized as exceptionally efficient, and thus commercially recommended by the Commonwealth Scientific and Industrial Research Organization (CSIRO) since 1968. The strain is a subculture of USDA 136, which was derived from USDA 122 (Dr. Peter van Berkum, personal communication]. Dr. Don Norris sent the strain from Australia to Dr. Johanna Döbereiner in Brazil in 1966, where its good symbiotic performance was confirmed (Döbereiner et al., 1970), and it was then recommended for use in commercial inoculants in 1977. However, poor nodulation with cultivar IAC-2, which was broadly used at that time, led to withdrawal of this recommendation in the following year (Santos et al., 1999; Hungria et al., 2006a).

In Brazil, with the crop expansion in the late 1960s, strains and commercial inoculants were brought in from the United States and Australia to be tested. *B. japonicum* strain SEMIA 566 was one of the first strains "selected" in Brazil. It was isolated from a nodule on the cultivar Hardee, after inoculation with material distributed by Dixie Inc., probably an inoculant from Nitragin (Milwaukee, USA); the strain belongs to serogroup 123. It was commercially recommended from 1966 to 1978, and greatly contributed to the successful establishment of the crop in the southern region of Brazil (Freire et al., 1983; Santos et al., 1999; Hungria et al., 2006a). A second strain, *B. elkanii* SEMIA 587, was isolated in 1967 from soybean nodules collected at Santa Rosa, Rio Grande do Sul (Professor Jardim Freire, personal communication]; it proved to be effective and competitive in a number of field trials and was used in commercial inoculants from 1968 to 1975, and again since 1979 (Santos et al., 1999; Hungria et al., 2006a). Another promising strain, *B. elkanii* 29W (=SEMIA 5019), was isolated in Brazil in 1979 from a nodule on the soybean line IAC-70-559 (Peres, 1979). Unfortunately, there is no information on the inoculant used in the area (Dr. Jose

Roberto Peres, Embrapa, personal communication]. The strain proved to be efficient and competitive in a newly cultivated area in central Brazil in the 1970s, the Cerrado region, now the main grain-producing region of Brazil. Since 1979, the combination of *B. elkanii* strains SEMIA 587 and 29W has been broadly used in commercial inoculants and represents a major factor in the establishment of soybeans in the Cerrado (Peres, 1979; Peres and Vidor, 1980; Hungria et al., 2006a).

Later, higher N demands of more productive soybean genotypes led to strain-selection programs, with the goal of reaching greater capacity for N_2 fixation, higher competitiveness in soils with established populations of rhizobia, and tolerance of frequently stressful tropical conditions. The approach used in Brazil was successful and may be applied in other countries. Without natural biodiversity from which to select new strains, one approach has been the isolation of efficient and adapted naturalized strains from areas previously inoculated with strain SEMIA 566. The first step in the selection was the isolation of rhizobia from large pink nodules on field-grown soybean plants. Each of these rhizobial isolates was then tested for N_2 fixation capacity under laboratory and greenhouse conditions (plant growth and competitiveness), and then in field trials (Peres et al. 1984). After several cropping seasons, the isolate CPAC 15 (=SEMIA 5079) was identified, and shown to statistically increase soybean yield in comparison with strains SEMIA 587 and 29W, which were used in commercial inoculants (Vargas et al., 1992; Peres et al., 1993; Hungria and Vargas, 2000; Hungria et al., 2006a).

A second strain-selection approach, initiated simultaneously, tested individual colonies from pure cultures of strain CB 1809 for variants that were of greater efficiency in BNF and also highly competitive. When dozens of individual colonies of CB 1809 were separately evaluated for BNF and plant growth under greenhouse conditions, significant variation in these traits was evident and the best strains were taken to field trials, and re-isolated from the nodules of plants showing the best symbiotic performance and grain yield, to improve the adaptability of the isolates to the local edaphoclimatic conditions. This approach led to the identification of *B. japonicum* (now *B. diazoefficiens*) strain CPAC 7 (=SEMIA 5080) (Vargas et al., 1992; Peres et al., 1993; Hungria and Vargas, 2000; Hungria et al., 2006a).

Both CPAC 7 and CPAC 15 were included in the list of strains authorized for use in soybean commercial inoculants in 1992 and are currently very successful and preferentially used by farmers in the Cerrado region. Noteworthy is the natural variability that may be found in each strain (Torres et al., 2012) (Fig. 99.4), which can be used to identify new, elite strains.

As commented before, in Argentina, soils are also devoid of indigenous soybean *Bradyrhizobium*, and imported inoculants and strains were brought for evaluation in areas cropped with soybean for the first time. The search identified *B. japonicum* strain E109 (=USDA 138, =SEMIA 5085, =TAL 377, serogroup 6) as being effective, and this strain has been used for decades in the majority of the commercial inoculants used in Argentina.

In China, an important producer (Fig. 99.1) and the main soybean consumer, inoculants are barely used (Ruiz-Sainz et al., 2005), whereas in Paraguay and other countries of South America imported inoculants carrying Argentinian and Brazilian strains are broadly used. In India, despite the presence of numerous producers of inoculants, information is scarce on their use with soybean; interestingly, more emphasis is given to plant growth-promoting bacteria and to compost use (Patil et al., 2006).

Another interesting recent observation from Canada has shown that indigenous bradyrhizobia symbionts of native legumes can adapt to nodulate soybean, representing a novel source of strains (Tang et al., 2012). High rates of horizontal and vertical transference of nodulation genes to indigenous bradyrhizobia have also been reported (Batista et al., 2007; Barcellos et al., 2007; Tang et al., 2012), resulting in new strains with the capacity of nodulating soybean. However, we are far from understanding the long-term impacts of these genetic events on BNF performance under field conditions.

In addition to slow-growing *Bradyrhizobium* spp., soybean is also nodulated by fast-growing rhizobia found in soils of the People's Republic of China (Keyser et al., 1982; Thomas-Oates et al., 2003), belonging to the genus *Sinorhizobium* (=*Ensifer*). Originally, it was reported that these fast growers were specific for Asian soybean lines (Keyser et al., 1982; Devine, 1985). However, later it was shown that nodulation and BNF with fast growers can occur also in modern soybean genotypes, such as those produced in the United States (Balatti and Pueppke, 1992) and Brazil (Chueire and Hungria, 1997). Advantages of using fast-growing rhizobia with soybean potentially include greater facility of commercial production, more rapid establishment in soils, displacement of indigenous *B. japonicum* strains and easier manipulation of genes; consequently, strain selection took place (Buendía-Clavería and Ruiz-Saínz, 1985; Chatterjee et al., 1990; Buendía-Clavería et al., 1994). However, a main limitation has been that the competitiveness of *S. fredii* is related to pH; *S. fredii* is more competitive than *B. japonicum* in alkaline soils, whereas the converse applies in acid soils (Buendía-Clavería et al., 1994; Hungria et al., 2001). Since most soils cropped to soybean are acidic, the potential utility of fast growers as inoculants is limited.

In several countries, there is no legal requirement for specifying the strains in inoculants (e.g., the United States]. However, DNA fingerprinting of strains isolated from inoculants that do not specify the contents indicates the presence of USDA 110, USDA 122, and 532C. It is also possible, in some countries, to patent strains, and some companies have registered strains with specific superior

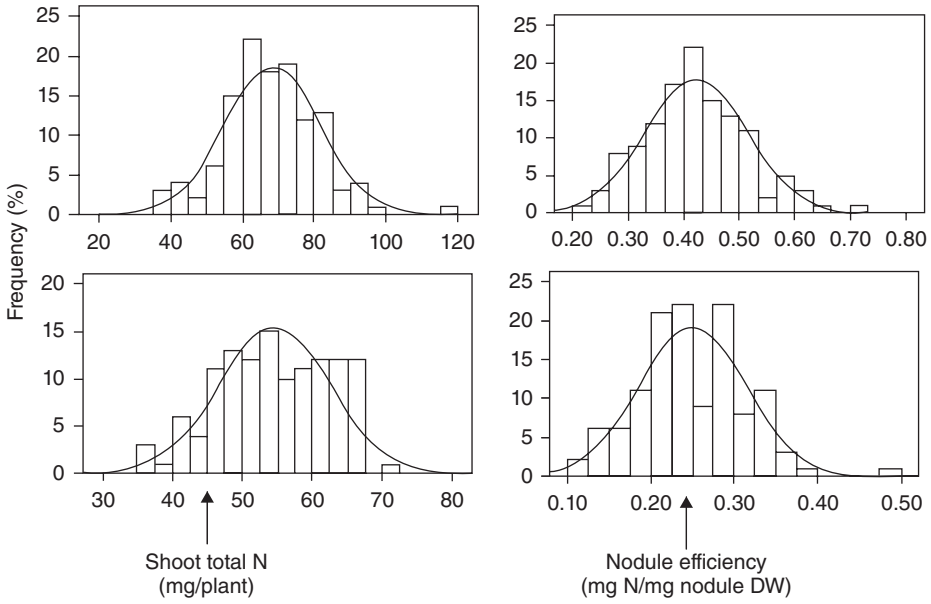

Figure 99.4 Natural variability in shoot total N and nodule efficiency of soybean nodulated by 40 variant and parental strains of *B. japonicum* CIAT 89 and CIAT 104. Arrows indicate the values referring to the parental strain. (Source: Adapted from Torres et al. (2012).

properties. One example is an "improved" *B. japonicum* released by researchers of the Agricultural Research Service [ARS]-USDA in the United States. In 1991, Dr. L. D. Kuykendall and Dr. W. J. Hunter patented a strain with a performance superior to those being used by farmers and licensed it to the Urbana Laboratories of Missouri. They called the process "direct selection," which included simple screening of strains. Nitrous acid was used to trigger gene mutations in a liquid culture of *B. japonicum* strain I-110, and again with "direct selection" they isolated a mutant called TA11 Nod+ in 1994 (USDA-ARS, 2002). Recently in the United States, a patent was granted for a herbicide-resistant inoculant strain (ABM201, =ATCC PTA-10253); this natural mutant – which tolerates glyphosate – was also selected from a "traditional" *B. japonicum* strain (Kuykendall, 2012).

In conclusion, it is noteworthy that the large market for inoculants for soybean relies on very few strains that were identified – or are derived from strains identified – long ago. Probably more than 90% of inoculants produced globally include strains USDA 110 (identified in the late 1950s], 532C [1980s], four "Brazilian" strains [selected or derived from strains selected in the 1960s and 1970s], and the "Argentinian" E109 [derived from USDA 136, also from the 1970s]. These few strains may be variously present in the more than 80 million doses of inoculants produced annually. Possibly, this very narrow bacterial germplasm results from the lack of biodiversity in the main producing and inoculant-consuming countries or from difficulties or lack of interest in exchanging germplasm with domestication centers. It is noteworthy that these "old" strains show high efficacy in conjunction with newly released, more productive soybean cultivars (e.g., Fig. 99.5). Indeed, there are reports of BNF sustaining yields higher than 6000 kg ha^{-1} (Hungria et al., 2006a). The following questions arise: is there a limit to BNF by those strains? Or will the source–sink demand of the soybean–*Bradyrhizobium* symbiosis (Kaschuk et al., 2010) limit BNF rates? Compelling evidence of source–sink control of N supplied by the strains available was obtained in a recent experiment: when soybean density was decreased from 320,000 to 80,000 plants/ha, the rates of BNF were increased four-fold and supported statistically similar total yields de Luca et al. (2014). These strains may have the potential to supply the N demands of soybean realizing their genetic potential, estimated at approximately 8000 kg ha^{-1} (Specht et al., 1999). This implies the potential to supply the astonishing amount of 640 kg N ha^{-1} per growing season. Accordingly, strain-selection programs should be directed not at increased BNF capacity, but at compatibility with fungicides, insecticides, and herbicides that are heavily used in soybean cropping and which can affect rhizobial viability on seeds and in soil (e.g., Campo et al., 2009).

If more efficient strains are needed in the future, new searches in centers of genetic origin or domestication of soybean may be profitable. Indeed, high genetic diversity of soybean rhizobial strains has been reported in China (Keyser et al., 1982; Yang et al., 2006; Man et al., 2008), as well as in other countries including Japan (Minamisawa, 1989) and Thailand (Yokoyama et al., 1999). Certainly, very effective strains are likely to be present within this biodiversity. In addition, microevolution of bradyrhizobia should be considered (Tang et al., 2012).

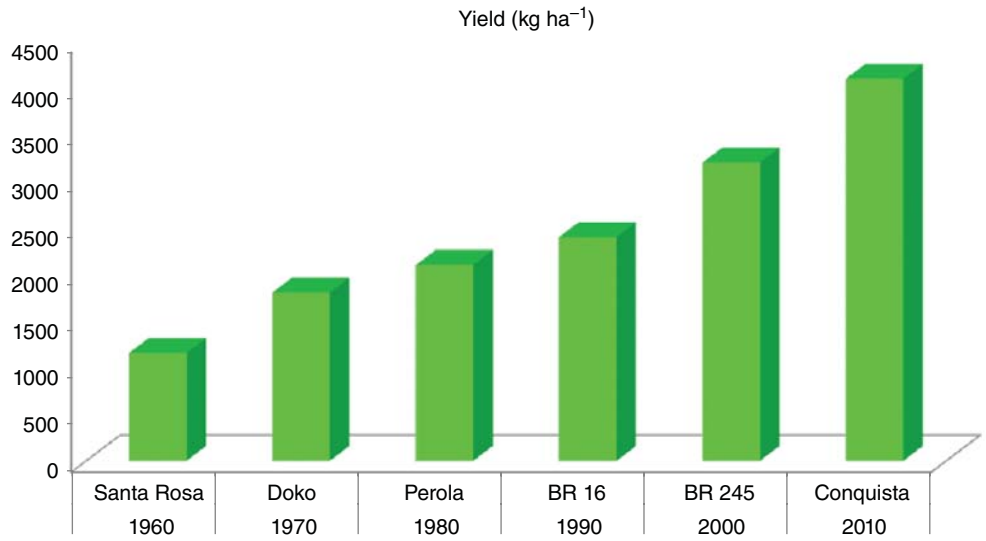

Figure 99.5 Effect of inoculation with *B. elkanii* strain SEMIA 587 in areas void of naturalized population on soybean yield of popular cultivars from each decade in Brazil.

99.1.3 How Limiting is Competitiveness with Soil Populations and How Effective can Reinoculation be?

Competitiveness can be defined as the ratio between the number of bacteria of a particular strain in the inoculum and the percentage of nodules containing the strain on the roots of the host plant. It has been long considered as a major limitation to BNF, where the success of inoculation depends on the ability of the inoculant strain(s) to compete with infective but ineffective strains established in the soil (Nicol and Thornton, 1941).

Through decades, several laboratories have focused their efforts on identifying factors that influence rhizobial competitiveness. These factors include mobility and chemotaxis (e.g., Caetano-Anolles et al., 1988; Catlow et al., 1990; Zdor and Pueppke, 1991), cell-surface polysaccharides (e.g., Bhagwat et al., 1991; Zdor and Pueppke, 1991), bacteriocin production (e.g., Triplett, 1990a,1990b), infection rate (e.g., Hahn and Hennecke, 1988), and the ability to respond to various substrates (e.g., Bottomley et al., 1990). However, the subject is far from being clarified, most probably because superior competitiveness may result from a combination of several bacterial characteristics. It should be mentioned that properties such as early nodulation (Oliveira and Graham, 1990) or adaptation to soil conditions (Hungria and Vargas, 2000; Hungria et al., 2006a) lend competitive ability.

Competitiveness has been considered a serious limitation to the introduction of superior inoculant strains; poor responses to inoculation have been reported even when the numbers of indigenous rhizobia are low (Weaver and Frederick, 1974; Singleton and Tavares, 1986; Thies et al., 1991a,1991b). In the case of soybean, many strains survive saprophytically for prolonged periods, and hence repeated cultivation of the legume promotes long-term establishment of strains introduced via inoculants and on seeds. Accordingly, increased bradyrhizobial populations over time have been reported worldwide, for example, in Australia (Brockwell et al., 1987), the United States (McLoughlin et al., 1990), Argentina (Brutti et al., 1998), and Brazil (Mendes et al., 2004).

The most discussed example of the "competition problem" is that of strains related to the 123 serogroup in the Midwest of the United States (Keyser and Cregan, 1987), with reports of occupations of 60 to 80% of the nodules by indigenous rhizobia (Kvien et al., 1981). Serogroup 123 has also been reported in Canada (Semu and Hume, 1979) and Korea (Kang et al., 1991), and, in Brazil, it is established in practically all cropped areas, due to the introduction of two serologically related inoculant strains, SEMIA 566 and CPAC 15 (Vargas et al., 1994; Ferreira and Hungria, 2002; Mendes et al., 2004; Hungria et al., 2006a). What makes 123-related strains so competitive remains to be elucidated. Gaining an understanding of competitiveness genes in these strains might be of great benefit.

High numbers of cells in inoculants and/or application of highly competitive strains are needed to outcompete resident soil populations. Results from a classical study by Weaver and Frederick (1974) indicate that the concentration of the inoculant strain should be at least 1000 times higher than the soil rhizobial population to result in 50% nodule occupation. Other reports have indicated that, in the presence of populations as low as 10 to 20 cells/g of soil, it is not possible to obtain significant occupancy by a new strain (Singleton and Tavares, 1986; Thies et al., 1991a,1991b).

Consequently, inconsistent responses to inoculation have been attributed to the indigenous or naturalized population, to the enrichment of the indigenous population due to prior cropping of legumes, and/or to a combination of both factors (e.g., Herridge et al., 1987; Sadowsky and Graham, 1998; Thies et al., 1991a,1991b; 1995). This is still the case in the United States. A recent analysis of 73 experiments conducted in Indiana, Iowa, Minnesota, Nebraska, and Wisconsin, by de Bruijin et al. (2010) indicated that inoculation offers limited success for either a yield increase or improved economic return on Midwestern soils previously cropped with soybean.

However, it is intriguing that this is not the case in South America, where more than 150 experiments have consistently proved that annual reinoculation is worthwhile. Edaphoclimatic conditions in Brazil and in many areas of South America commonly include high temperatures, frequent periods of drought, and acidic, infertile soils (Hungria and Vargas, 2000). Therefore, despite showing a high population of soybean bradyrhizobia – reaching up to 10^6 cells/g of soil due to soybean cropping – it is possible that this population is at a low metabolic level and needs some time or another factor to be primed and "ready" for nodulation. A common observation is that, in "old" areas, inoculated soybeans show profuse nodulation at the root crown whereas non-inoculated plants do not, as shown in Figure 99.6. This early root-crown nodulation fosters prompt establishment of more vigorous plants and is responsible for yield increases observed in Brazil, Argentina, and other South American countries when annual reinoculation is practiced. In non-inoculated plants, delayed infection results in nodules present at least 1–2 cm below the crown.

In Brazil, the analysis of 80 field trials, all producing more than 2000 kg ha^{-1} of grain, performed in northern Brazil (Boa Vista, State of Roraima, 02°49'S–60°40'W') to the southern states (Passo Fundo, State of Rio Grande do Sul, 28°15'S–52°24'W) – all in soils previously cropped with soybean and showing 10^3 to 10^6 cells/g of soil – has shown mean increases of 8% in yield due to annual reinoculation. It is remarkable also that no yield gains were obtained with the application of 200 kg N ha^{-1}, highlighting the efficiency of the BNF process (Fig. 99.7). It is also noteworthy that a preliminary analysis of nodule occupancy in several of these trials indicated that, for an efficient strain, nodule occupancy of around 35% can supply the crop's needs, less than the 50% recommended by Frederick and Weaver (1974).

Figure 99.6 Nodulation at the root crown, associated with inoculant applied to seeds in an area "traditionally" cropped with soybean in Brazil.

Similar results have been observed in the analysis of ten consecutive soybean crops (from 2000 to 2010] in "traditional" cropping areas of the State of Mato Grosso do Sul (centrally located Dourados is at 22°13'S and 54°48'W] in western Brazil) (Mercante et al., 2011). By the end of the tenth year, a cumulative yield gain of 2754 kg ha^{-1} resulted from reinoculation. Therefore, as pointed out by the authors, by reinoculating every year, the farmer would garner a profit of 1 crop per every 10 crops (Mercante et al., 2011).

Similar results have been reported in Argentina, and, in an analysis of 62 trials, increases due to reinoculation ranged from 2.1 to 20%. In these trials, the weighted mean yield of the reinoculated treatment was 3133 kg ha^{-1}, and the increase due to reinoculation averaged 11% (Hungria et al., 2006a).

Another important benefit reported from reinoculation in Brazil is the residual nitrogen left for the following crop (Hungria et al., 2005; 2006a; 2007). Experiments were performed to evaluate this contribution in areas "traditionally" cropped with soybean, in Londrina (23°18'S–51°09'W: 10^6 cells of *Bradyrhizobium*/g soil), by comparing two treatments, non-reinoculated and reinoculated. In 9 years, mean increases of wheat [*Triticum aestivum* L.] grain production after reinoculated soybean averaged 14%. For short-season maize [*Zea mays* L.], after 4 years, the mean yield increase averaged 12%.

Finally, another important benefit from reinoculation is the gradual replacement of persistent strains with more efficient counterparts. Mendes et al. (2004) performed an experiment in Planaltina (15°27'S–47°37'W), central Brazil, in which, in the first year, a soil void of soybean bradyrhizobia was inoculated with serologically distinct strains: CPAC 15 (serocluster USDA 123), highly competitive, and CPAC 7 (serogroup of CB 1809), which is less competitive than CPAC 15 but efficient in fixing nitrogen. In the second year, each of the initial plots was subdivided and either inoculated or not, with CPAC 7 or CPAC 15, and in the third year, the entire area was inoculated with CPAC 7. In the treatments where strain CPAC 7 was used as inoculum in each of the 3 years, its representation in nodules increased to 70% in the third year. In the treatment first inoculated with CPAC 15, and the two following years with CPAC 7, nodule occupancy by CPAC 7 in the third year was also high, at 62%. However, in the treatment inoculated with CPAC 7 in the first year

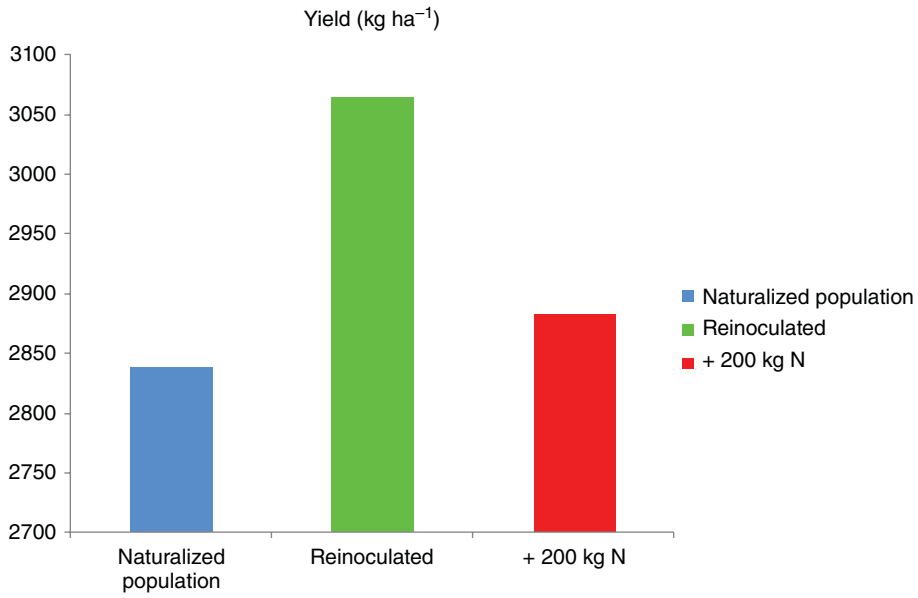

Figure 99.7 Comparison of treatments non-inoculated, inoculated and non-inoculated receiving 200 kg N ha^{-1} [split twice, at sowing and broadcast at flowering]. Mean of 80 field trials performed in Brazil, in soils previously cropped with soybean, and with indigenous bradyrhizobial populations ranging from 10^3 to 10^6 cells/g soil.

and without further reinoculation, nodule occupancy by this strain decreased to only 8%. Therefore, annual reinoculation is key to both the substitution of dominant serogroups and to guaranteeing the persistence of elite strains (Mendes et al., 2004).

The question that remains is the reason for the absence of positive effects of reinoculation in North America, as opposed to South America, where significant benefits are observed. As we pointed out earlier (Hungria et al., 2006a), possible explanations for the general lack of response to inoculation in soils containing indigenous bradyrhizobia in the United States may include higher competitiveness of the bradyrhizobial strains established in North American soils, and also lack of emphasis on the need for reinoculation, including the need for care in mixing inoculant cultures and seed protectants. In fact, yield responses to reinoculation of up to 12% were reported in US northern and eastern soybean production states within the Midwest, whereas states from the Great Plains region did not show consistent yield responses to reinoculation of "old" fields (Abendroth et al., 2006). It seems that, in northern states, these responses were more related to cold spring temperatures that limit growth and multiplication of *B. japonicum*, and in eastern states the extensive use of seed-applied fungicides may be partly responsible for the observed yield responses to reinoculation in "old" soybean fields. In South America, the possibility exists that soil rhizobia are less competitive and that environmentally stressful conditions dictate reinoculation to ensure early nodulation and benefit early crop growth. Credit should go to the farmers who believe in and use inoculants every year.

99.1.4 N-fertilizers × Biological Nitrogen Fixation with Soybean: Who Wins?

Contributions of BNF are considerably affected by the availability of mineral N, and as developed countries use more N-fertilizers, the contributions of BNF in these countries are generally lower when compared with those adopting low-N inputs, as in South America.

In an interesting analysis, van Kessel and Hartley (2000) examined results generated in 362 trials – mainly in the United States and Australia over a 25-year period – in which ^{15}N-isotopic dilution or N difference with non-nodulating isolines methods were used to estimate contributions of BNF to soybean. In this data collection, soybean derived an average of 59% of its N from BNF; this value is similar to the 53% reported for 33 commercial soybean fields in Australia (Unkovich and Pate, 2000). A conclusion from the study was that the %N derived from BNF declined from 65% [analysis of 196 trials] to 54% [166 trials] since 1985; the authors attributed the lower symbiotic performance to increased use of N-fertilizers.

In northern and central China, the center of genetic origin of soybean, N-fertilizers are also applied, and the best results were obtained with top dressing [50 kg N ha^{-1}] at the V2 stage, and, especially, at early flowering (R1] (Gan et al., 2002; 2003). These authors mentioned that inefficient use of N-fertilizer often results in high costs, and it is worth mentioning that the yields were lower than in the Americas.

Soybean cropping in Africa is dramatically changing, with adoption of new genotypes, cropping systems, and inoculants; therefore, there is need for new quantification studies. For now, on searching the literature, Ronner and Franke (2012) found contributions of BNF ranging from 3 to 89%, and in some evaluations performed in the N2Africa project (small farmers with low inputs) the average for inoculated soybean was 70%.

In Argentina soybean inoculated with strain E109, without N-fertilizer, achieved 6000 kg ha^{-1} (Hungria et al., 2006a), and in Londrina, Brazil, soybean yielded 5890 kg ha^{-1} again without N-fertilizer (Zotarelli, 2000). Still in Brazil, a survey of studies using ^{15}N isotopic dilution, δ^{15}N, N balance, and the N-ureide technique indicated that the N derived from BNF ranged from 69 to 94%, with estimated rates reaching 300 kg N ha^{-1} (Hungria et al., 2005). The contributions of BNF may be considerably higher, as root N was not considered in these studies; it may represent 30 to 35% of the total plant N (Peoples and Herridge, 2000; Khan et al., 2002).

The comparison made by van Kessel and Hartley (2000) and the results obtained in South America emphasize the importance of management to maximize the contribution of BNF. However, there is strong pressure in South America and in other countries from fertilizer companies to apply chemical N to soybean. The stated objective is to achieve higher yields, or to attend to specific conditions, such as early short season cultivars, indeterminate genotypes, and soils with low-N levels.

One idea that has been discussed for decades has been of the use of "starter" N to overcome problems related with N immobilization, mainly when soybean is cultivated after a non-legume crop, and before BNF is fully established. However, in Brazil dozens of experiments have shown that N doses as low as 20–40 kg N ha^{-1} may drastically decrease nodulation and BNF, with no benefits to yield (e.g., Mendes et al., 2003; Hungria et al., 2005; 2006a,2006b; 2007). Figure 99.8 shows one example of a combination of 20 field trials, with no response to 30 kg N ha^{-1} as starter N.

Experiments have also been performed to confirm that no extra benefits would be obtained by the application of N-fertilizer throughout the plant-growth cycle, because doubts were raised about the capacity of BNF to provide N after flowering, due to nodule senescence (Hungria et al., 2006b; Mendes et al. 2008). Again, no benefits were observed with the supplementary dose of 50 kg N ha^{-1} at the R2 or R4 stages (Fig. 99.8). In the experiments shown in Figure 99.8, BNF contribution evaluated by the N-ureide technique dropped from 84% on the reinoculated treatment to 77 and 78% when N was applied at R2 and R4, respectively, and to 44% with the application of 200 kg N ha^{-1} (Hungria et al., 2006b).

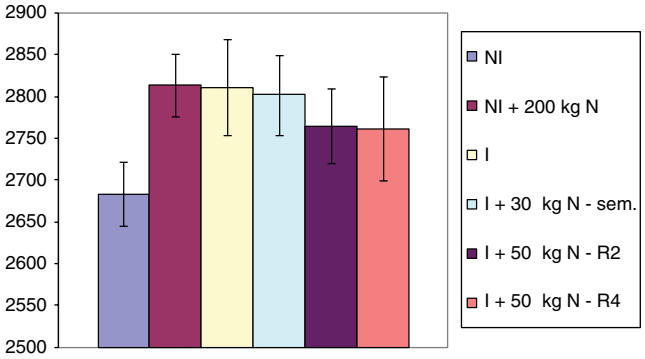

Figure 99.8 Soybean yield (kilogram per hectare) in soils with established populations of *Bradyrhizobium* (>10^3 cells/g) in treatments non-inoculated (NI), non-inoculated receiving 200 kg N ha^{-1} (split at sowing and at flowering – R2), and inoculated (I), with and without N-fertilizer applied at sowing (30 kg N), at full flowering-R2, or at the grain-filling stage-R4 (50 kg N ha^{-1}). Averages of 20 trials performed in the State of Paraná, Brazil. Columns followed by the same letter do not show statistical difference (Duncan, $p \leq 0.05$). Adapted from Hungria et al. (2006b).

First in Argentina and now in Brazil, in the last few years there has been a preference for cultivars of indeterminate habit, in which vegetative growth occurs mainly after flowering. Concerns have been raised about a decrease of BNF after flowering, as two thirds of the plant's growth occurs after this period. But again, in a series of experiments performed for the last 2 years, we have consistently observed that these cultivars also do not need any N-fertilizer supply (e.g., Fig. 99.9). It is noteworthy also that the inhibition of nodulation and BNF, resulting in less yield (Fig. 99.9) by application of chemical N was stronger with cultivars of the indeterminate type. Finally, in a comparison of 20 experiments with cultivars of short- and long-growth cycles, Hungria et al. (2006b) also reported that there was no benefit in yield by adding N-fertilizer at sowing, R2 or R4. Indeed, lack of response to N-fertilizer has been observed in doses of up to 400 kg N ha^{-1}, split 10 times throughout the plant's growth cycle (Nishi and Hungria, 1996; Hungria et al., 2007) Regarding the use of late supplemental N-fertilizers on soybeans it is also important to mention the findings of Mastrodomenico and Purcell (2012) who demonstrated that, under water-replete conditions, BNF can continue late into pod-fill and provide up to 90% of the N content in the seeds. Among soybean isolines for maturity, they did not find differences in seed yield or N content at maturity and also observed that for the later maturing genotypes, a larger reservoir of N in the vegetative tissue was not fully used, and as a consequence, N was not a limitation to yield.

From our discussion in this section we may conclude that the success of BNF with soybean is closely related to soybean breeding programs that rely on inoculation and also to the selection of bradyrhizobial strains with superior

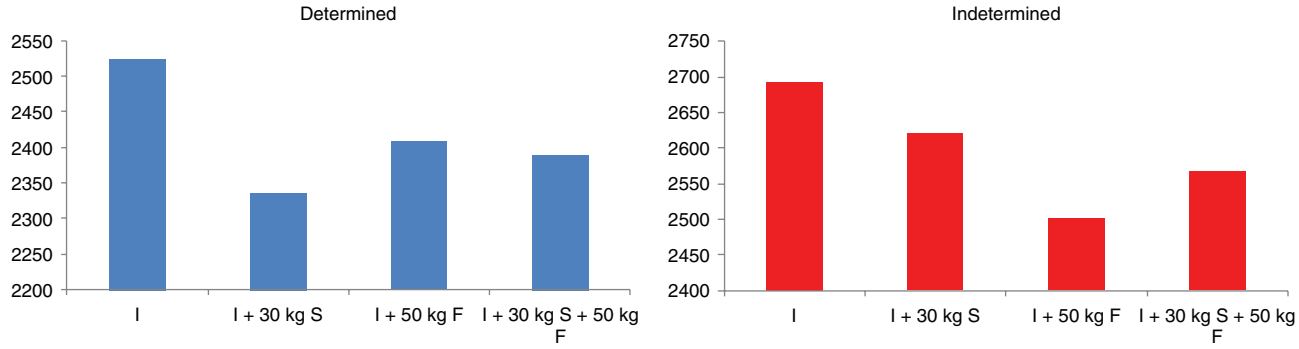

Figure 99.9 Grain yield (kilogram per hectare) of soybean of determinate and indeterminate type of growth when reinoculated with or without a supply of N-fertilizer at sowing (S) and/or at full-flowering stage, R2 (F). Experiment performed in a soil with 10^6 cells of *Bradyrhizobium*/g soil. (M Hungria and M.A. Nogueira, unpublished data).

nitrogen-fixing capacity. In countries where BNF has not been a main goal in breeding programs, yield increases can be obtained with the addition of N-fertilizers. On the contrary, where BNF is continually monitored in breeding programs, the results obtained so far indicate that high yields can be obtained, relying mainly on BNF.

99.1.5 Main Constrains to BNF with Soybean

Several concerns underpin maximizing BNF by soybean in the next decade. Lack of emphasis on BNF in soybean breeding programs is already a reality in many developed countries and may expand to developing countries with the increasing presence of private companies interested in selling N-fertilizers.

Care should also be taken in relation to management practices. Application of fungicides to seeds to which an inoculant is then applied can adversely affect rhizobial cell number (e.g., Hungria et al., 2006a; 2007, Campo et al., 2009). Pre-inoculation of soybean seed, especially when fungicides and/or insecticides are applied, can be disastrous, decreasing nodulation drastically (Fig. 99.10). Attention should also be paid to global climate changes, as the symbiosis is sensitive to high temperatures and water deficiency.

Nutrition and pest control are site-specific problems. A generally applicable constraint is lack of extension outreach to explain the benefits of BNF to farmers.

99.1.6 Economic, Social and Environmental Implications of BNF by Soybean

Economic returns of BNF with soybean in South America are outstanding, with the Brazilian case used here as an example. Considering that for each 1000 kg of soybean produced, the

Figure 99.10 Nodulation of soybean under field conditions when (a) pre-inoculated or (b) inoculated on the same day. Photo: Agronomist José Rogério dos Santos.

plant requires 80 kg of N, the crop needs 240 kg N ha^{-1} to achieve the national mean yield of 3000 kg ha^{-1}. Generally, Brazilian soils are poor in N and usually supply only 15 to 30 kg N/crop, in which case the plants need an additional 210–225 kg N ha^{-1}. As N-utilization efficiency is around 50%, the N application need is doubled, to ≈420 kg N. At US$ 1.4/kg N at the local market(urea, the cheapest source), the cost of applying N-fertilizer would be ≈US$ 15 billion nationally, for the 25 million ha cropped in 2012/2013. A similar picture is found in other countries of South America; clearly, the continent could not produce half of the world's yield of soybean without BNF.

The economy related to soybean agribusiness affects thousands of farmers and attendant industry workers, with far-reaching social implications. Similarly, soybean and BNF profoundly affect small-holding farmers and the economies of several African countries as exemplified in the project N2Africa (http://www.n2africa.org.br). There is no

doubt that, in these countries, there is potential for BNF to play increasingly important roles in ensuring food security and improving life quality.

The environmental effects of using N-fertilizers should not be forgotten. Pollution of aquifers, rivers, reservoirs, and lakes is associated with leaching and run-off of nitrate. In addition, the emission of greenhouse gases related to N-fertilizers is an important issue. Brazil again is a good example, as the government launched a program called "ABC" (Agriculture of Low Carbon Consumption) and among the targets is the adoption of agricultural practices involving BNF, with the commitment of an area expansion of 5.5 million hectares with a mitigation potential of 10 million tons of CO_2. Considering the synthesis, transport, and application of N-fertilizers in Brazil, Hungria et al. (2013) used a rate of 4.5 kg of e-CO_2/kg (CO_2 equivalent) of N-fertilizer and came to the conclusion that if nitrogen-fixing bacteria were not used for the soybean crop, the application of N-fertilizer would result in the emission of about 45 million tons of e-CO_2.

99.2 CONCLUDING REMARKS

High rates of BNF are reported worldwide for the soybean–*Bradyrhizobium* symbiosis, confirming a high capacity to supply N needs to reach high yields. This "perfect" symbiosis should be used as a model in the search for genes related to the efficiency of the biological process. Strategies such as annual reinoculation, plant breeding in the absence of N-fertilizers, and strain selection should be considered in all countries cropping the legume. The results reported in this chapter highlight the fact that BNF by soybean is a key ecological service that contributes billions of dollars to the global economy, increasing farmers' profits and helping to ensure food security. Particularly important is the fact that BNF is applicable and relevant to all kinds of management practices, from large farmers adopting modern technology in the Americas to small-holding farmers in Africa contributing to local economies. Environmental impacts of BNF by soybean are also extremely important, such that if no attention is paid to the contributions of BNF to soybean crops, increases in the applications of N-fertilizers to the 103 million ha cropped worldwide would substantially increase emissions of greenhouse gases.

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Chapter 100

Nodule Functioning and Symbiotic Efficiency of Cowpea and Soybean Varieties in Africa

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100.1 INTRODUCTION

Biological N_2 fixation still remains the most sustainable technology to contribute N to both agricultural and natural ecosystems. Members of the plant family Leguminosae have the unique ability to form N_2-fixing symbioses with root-nodule bacteria ("rhizobia") belonging to the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* (*Ensifer)*, *Mesorhizobium,* and *Azorhizobium*. Inside root nodules, these rhizobial bacteria are able to reduce atmospheric N_2 into NH_3 using the enzyme nitrogenase (Dakora, 1994). The NH_3 produced in excess of the bacterial requirement is secreted into host plant cells, where it is converted into amino acids and other nitrogenous solutes via the GS/GOGAT pathway for plant use. Nitrogenous solutes are thus exchanged by bacteria for photosynthate produced by the host plant, and this forms the basis of the mutualism between root-nodule bacteria and members of the Leguminosae. This review is an attempt to summarize recent data on nodule functioning, root-nodule biodiversity, and symbiotic N contribution of cowpea and soybean used in African agriculture.

100.1.1 Constraints to Cowpea and Soybean Production in Africa

While this symbiotic relationship between soil bacteria and cowpea/soybean plays a major role in sustaining the N economy of agricultural ecosystems, tapping it for increased food security in Africa has remained a challenge. This is due largely to many constraints, which include lack of high yielding legume genotypes/varieties, absence of suitably selected strain/genotype combinations for enhanced N_2 fixation and grain yield, and little information on the biodiversity and N_2-fixing ability of bacteria nodulating African food grain legumes. Eliminating these limitations is crucial for improving N_2 fixation and the agricultural yield of symbiotic legumes.

With 23% protein and 57% carbohydrate in the grain, cowpea is the most important food legume in tropical Africa. Both its leaves and grain are eaten as food, and together they constitute an important part of the African diet. Globally, cowpea is also cultivated for use as food in Brazil (see Chapter 99), India, and the Southern United States.

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Because of its high N_2 fixation (Makoi et al., 2009; Belane and Dakora, 2009; Pule-Meulenberg and Dakora, 2009, Pule-Meulenberg et al., 2010, Belane and Dakora, 2011; Belane et al., 2011) cowpea leaves and grain are very rich in protein, and are commonly used as feed/fodder for livestock in Australia and the United States. Despite the important value of cowpea as food crop, little is known about the diversity of its nodule bacteria and symbiotic functioning.

Although it has its origin in Asia, soybean is now cultivated worldwide in Australia, Brazil, the United States, Argentina, and China. It is also cultivated in Africa, though it remains unclear when the crop was introduced into the African continent (Mpepereki et al., 2000). Of the 216 million tons of soybean produced worldwide in 2007, only 1.5 million come from Africa, an indication of its limited production. Its promotion as the leading commercial legume stems from the fact that the grain contains 40% protein and 20% oil, making it a high-protein food/feed for humans and livestock (Zarkadas et al., 2007). Increased soybean cultivation in Africa is however, constrained by lack of information on the legume's adaptability to African conditions, especially those bred to nodulate with local soil bacteria.

100.1.2 Nodulation, N_2 Fixation, and N Contribution by Cowpea and Soybean in Africa

Historically, cowpea has been known to form effective symbiosis with only slow-growing root-nodule bacteria (*Bradyrhizobium* species), even though fast growers were frequently isolated as contaminants from cowpea nodules (Dakora and Vincent, 1984). Today, there is evidence (Mpepereki et al., 1996; Zhang et al., 2007; Steenkamp et al., 2008; Pule-Meulenberg et al., 2010) that cowpea is nodulated by both slow- and fast-growing root-nodule bacteria (i.e., *Rhizobium* and *Bradyrhizobium* species). Although Zhang et al. (2007) found cowpea to nodulate with both *Rhizobium* and *Bradyrhizobium* strains in China, a few studies in Africa could detect only *Bradyrhizobium* species in cowpea nodules (Steenkamp et al., 2008; Pule-Meulenberg et al., 2010), indicating that the choice of microsymbiont by cowpea could be environment dependent. Sarr et al. (2009) have also reported the isolation of *Ralstonia* from root nodules of cowpea, although its N_2-fixing status was not clear. Unlike cowpea, soybean is capable of forming effective symbiosis only with strains of *Bradyrhizobium japonicum* (Jordan, 1982; Black et al., 2012), *Bradyrhizobium elkanii* (Kuykendall et al., 1992), *Bradyrhizobium liaoningense* (Xu et al., 1995), and *Sinorhizobium fredii* (Chen et al., 1988; see Chapters 18, 99). Except for South Africa, the soybean material grown by farmers in Africa (e.g., Ghana, Nigeria, Kenya, Zimbabwe, Ethiopia, etc.) is mainly of the TGx type, or tropical glycine crosses (Abaidoo et al. 2007), which have been bred to effectively nodulate with indigenous cowpea-type *Bradyrhizobium* species. As a result, data on the symbiotic performance of soybean in Africa are largely available on the promiscuous nodulating genotypes developed by IITA.

In Africa, these so-called promiscuous soybean genotypes have been found to derive between 46% and 78% of their N nutrition from symbiotic fixation and to contribute about 24 to 260 kg N ha^{-1} under field conditions (Table 100.1). Estimates of N_2 fixation by cowpea range from 4 to 201 kg N ha^{-1} across Africa (Sprent et al., 2010). Both soybean and cowpea are therefore capable of contributing significant amounts of N to the N economy of nutrient-poor soils in Africa, where N-fertilizer use is rare due to high cost, inaccessibility to resource-poor farmers, and its potential to pollute the environment. Furthermore, the greater N contribution by the two legumes also implies increased plant protein production, much needed for the diets of poor rural Africans.

100.1.3 Nodule Occupancy and IGS Type Symbiotic Efficiency in Cowpea and Soybean

The level of N_2 fixation in any legume is determined by the quality of infecting strains, as well as the photosynthate supplied by the host plant. Thus, good nodulation (i.e., high nodule numbers and dry weight) and enhanced N_2 fixation are the sole determinants of greater dry matter accumulation in symbiotic legumes. Studies carried out on six different promiscuous soybean genotypes in Ghana, and nine cowpea cultivars in South Africa, Botswana, and Ghana revealed marked variation in plant growth and grain yield as a result

Table 100.1 Estimates of N_2 fixation and N contribution by promiscuous-nodulating soybean genotypes in Africa

Country	%Ndfa	N-fixed (kg ha^{-1})	Method	References
Ghana	56–63	79–122	^{15}N natural abundance	Pule-Meulenberg et al. (2011)
Nigeria	46–54	51–78	Ureide-N technique	Osunde et al. (2003)
Kenya	47–67	53–82	^{15}N dilution	Kihara et al. (2011)
Nigeria	46–53	24–168	^{15}N isotope dilution	Sanginga et al. (1997)
Zimbabwe	55–78	160–260	N-difference	Kasasa et al. (1999)
Nigeria	52–68	60–108	N-difference	Ogoke et al. (2006)

Table 100.2 Diversity of strain IGS types isolated from root-nodules of cowpea genotypes grown at Wa in Ghana, and Taung in South Africa

Cowpea Genotype	Resident Strain IGS Type	
	Wa (Ghana)	Taung (South Africa)
Omondaw	VIII	I, II, VIII
Glenda	II, V, VII	III, V, VIII, XVIII
Apagbaala	III	III, V, VIII, XVIII
IT82D-889	V	III, V, VII, VIII
Bechuana white	V	II, V, VII, VIII
ITH98-46	VIII	III, V, VIII
Fahari		III, IV, VII, XIII, XIV, XVIII

(Source: BMC Microbiology, modified from Pule-Meulenberg et al., 2010)

of differences in symbiotic N_2 fixation (Belane and Dakora, 2009; 2010; Belane et al., 2011; Pule-Meulenberg et al., 2010; 2011).

More interestingly, PCR-RFLP analysis of cowpea nodules from the three countries revealed eighteen strain IGS types, with strong specificity to genotypes and geographical region (Table 100.2). Geographically speaking, strain IGS types I, IV, IX, X, XI, XIII, XIV, XVI, XVII, and XVIII were found only in nodules from Taung in South Africa, IGS types XV and XIX only in nodules from Glenvalley in Botswana, and IGS type XII only in nodules from Wa in Ghana (Pule-Meulenberg et al., 2010; see also Table 100.2). With regard to genotype specificity, strain IGS type I was found only in nodules of Omondaw, strain IGS types XV, XVI, and XVII only in nodules of Fahari, and strain IGS type VIII in all genotypes except Glenda (Pule-Meulenberg et al., 2010; see also Fig. 100.1). In Ghana, eighteen distinct IGS types were similarly found in root nodules of six field-grown promiscuous soybean genotypes (Pule-Meulenberg et al., 2011). There was, however, less strain IGS type/soybean genotype specificity, as strain IGS type II was isolated from all six soybean genotypes, and IGS types X and XI from five soybean genotypes (Pule-Meulenberg et al., 2011). While geographical specificities of strain IGS types can be easily understood, strain IGS type/host plant specificity is difficult to explain, and could be due to molecules involved in the early stages of nodule formation.

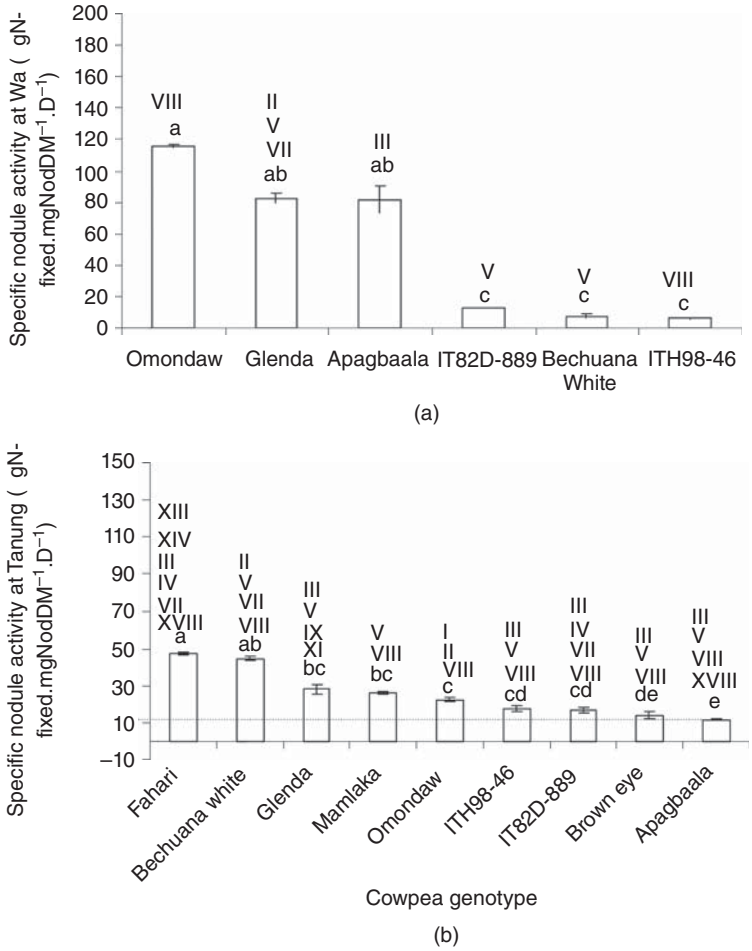

Figure 100.1 Specific nodule activity for nine cowpea genotypes grown at (a) Wa in Ghana, (b) Taung in South Africa. Bars with dissimilar letters indicate significant differences at $p \leq 0.05$. Numerals on the top of each bar represent the different IGS types (strains) that were found in the cowpea nodules from the particular genotype. (Source: BMC Microbiology, see Pule-Meulenberg et al., 2010.)

For the first time, the N_2-fixing efficiency of these IGS types was assessed by relating nodule function to nodule occupancy (i.e., to the identified strain IGS type resident in the nodule; see Pule-Meulenberg et al., 2010). The results showed large differences in symbiotic N produced by strain IGS types. But more importantly, in both soybean and cowpea, symbiotic promiscuity (i.e., multiple nodule occupancy or many strain IGS types in one nodule) appeared not to be a useful trait, as nodules with many bacterial occupants tended to produce less symbiotic N compared to nodules with sole occupants (Pule-Meulenberg et al., 2010; 2011). The differences in IGS type symbiotic efficiency were more likely due to the huge diversity of soil bacteria nodulating cowpea in Africa. For example, gene sequence analysis revealed clustering of cowpea nodule isolates with *Bradyrhizobium yuanmingense*, *Bradyrhizobium* sp. ORS 188, ORS 190 and USDA 3384, *Bradyrhizobium japonicum* USDA 38, and *Bradyrhizobium elkani* (Pule-Meulenberg et al., 2010). Some strain IGS types from Southern Africa also formed a unique group on the phylogenetic tree, which suggests a new *Bradyrhizobium* species (Pule-Meulenberg et al., 2010).

100.2 CONCLUSION

Both cowpea and soybean appear to be very promising grain legumes for food security in Africa. They derive a high percentage of N from symbiotic fixation for their N nutrition, and have the potential to contribute substantial amounts of symbiotic N to cropping systems in Africa. Cowpea seems capable of nodulating with a wide range of root-nodule bacteria in Africa, with some of them being closely related to soybean-nodulating bacteria. Whether with soybean or cowpea, nodulation promiscuity appeared less advantageous to the host plant in terms of symbiotic N yield. For example, one soybean genotype harboring as many as nine IGS types in its nodules in Ghana produced the least biological N.

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Chapter 101

Microbial Quality of Commercial Inoculants to Increase BNF and Nutrient Use Efficiency

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101.1 INTRODUCTION

The inoculation of crop plants is a "success story" of applied soil microbiology to provide a sustainable and effective source of nutrients to plants, particularly with regard to biological nitrogen fixation (BNF) and the development of rhizobial inoculants for legumes (Giller and Cadisch, 1995). Legume inoculation with rhizobia has resulted in improved crop productivity and soil fertility. It has been estimated that 21.5 Tg of N is annually fixed in agricultural systems through BNF (Herridge et al., 2008), and this is especially useful in situations where the natural N fixation is not optimal and/or exogenous N fertilizers are not applied (Kala et al., 2011; Stephens and Rask, 2000).

Plant growth enhancement has also been shown to occur beyond nitrogen fixation, and bacteria such as *Azospirillum* (see Chapter 90), *Azotobacter*, *Bacillus*, *Pseudomonas*, *Agrobacterium*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Streptomyces*, *Burkholderia* (see Chapters 17, 89), or *Serratia* have been shown to be beneficial soil

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microbes (de Bruijn, 2013). These plant growth – promoting rhizobacteria (PGPR) contribute to plant growth in different ways: some increase N uptake and synthesize phytohormones (auxin, cytokinin) which enhance root growth and facilitate minerals solubilization or iron chelation (Adesemoye et al., 2010; Collavino et al., 2010; Egamberdieva et al., 2010; Robin et al., 2008), while others are able to suppress soilborne pathogens by producing siderophores and antimicrobial metabolites, competing for nutrients and/or niches (Glick et al., 2007), or producing toxins that are inhibitory to the growth and/or activities of fungal and nematode pathogens of plants (Leifert et al., 1995; Pinchuk et al., 2002). As a result, inoculation with PGPR has been performed to improve seed emergence and plant growth and to increase yields of a wide range of crops (Dey et al., 2004; Herman et al., 2008; Minorsky, 2008; Muthaura et al., 2010; Wu et al., 2005), with the aim to decrease the dependence on chemical fertilizers, pesticides and supplements (Ashrafuzzaman et al., 2009; Bhattacharjee et al., 2008; de Bruijn, 2013; Lugtenberg and Kamilova, 2009; Vessey, 2003).

These PGPR include the free-living N-fixing bacteria which have been demonstrated to play an important role in the promotion of plant growth as they can fix significant amounts of atmospheric N (up to 60 kg N per ha and per year) in different cropping systems (Bürgmann et al., 2004). Inoculation of cereals with *Azospirillum* resulted in significant increases in plant biomass, nutrient uptake, tissue N content, plant height, leaf size and root length (Bashan et al., 2004; see Chapter 90). Kloepper and Beauchamp (1992) showed that wheat yield increased by up to 30% with *Azotobacter* inoculation and up to 43% with *Bacillus* inoculation. A number of genera have been identified as being able to convert unavailable P via the production of organic acids (Rashid et al., 2004), and these phosphate-solubilizing bacteria (PSB) will become increasingly important in the face of dwindling global supplies of affordably accessible P. Direct plant growth promotion has also been shown by strains of *Pseudomonas putida* and *P. fluorescens* that increased root and shoot elongation in canola (Glick et al., 1997) as well as in wheat and potato (Frommel et al., 1993).

The increasing demand for rhizobial and PGPR inoculants has resulted in commercial inoculant production facilities worldwide (Brockwell and Bottomley, 1995). New commercial inoculants are proliferating and all claim to substantially enhance the productivity of their various target crops. However, many of these products are sold without robust scientific data supporting their efficacy. Brockwell et al. (1995) reported that as many as 90% of all inoculants produced worldwide have no practical impact on target crop productivity. Ultimately, the efficacy of inoculants depends upon the quality to which they have been produced. If the quality is poor, then everything else is irrelevant (Herridge et al., 2002). Inoculant quality can be evaluated by a number of factors including the nature and effectiveness of the strain(s) it contains (Amarger, 2001), number of viable cells (Brockwell and Bottomley, 1995; Stephens and Rask, 2000), absence of significant contamination, effective and easy to apply formulation, adequate shelf life of the product, proper packaging and clear labeling with instructions for use on each package (Lupwayi et al., 2000; Xavier et al., 2004; see Chapter 97). However, so far, little attention has been paid to the quality of the inoculants during their production, leading to dramatically reduced effectiveness and consequently to a lower adoption by farmers. Since farmers cannot judge the quality of inoculant products at the time of purchase, there is often even less incentive for an inoculant producer to institute a quality control program, with the result that some producers sell poor quality inoculant products (Olsen et al., 1996; Singleton et al., 1997). Without a proper quality control program, the manufacturer is unlikely to have any more concrete information about the quality of his or her product than does the customer. When farmers realize that poor inoculant quality results in limited inoculant response, their confidence in the technology wanes, and both manufacturers and consumers are ultimate losers.

Consequently, the demand for a quality control system of the available commercial inoculants is vital and increasing. This notably involves the verification of the identity of the inoculant strain(s) and a check on the number of living cells in the inoculant (Tas et al., 1995; see Chapter 97). In particular, evaluation of inoculant quality by enumerating the viable cells present has been described as an accurate index of an inoculant's potential and of such significance in determining the effectiveness of inoculant that the need for quality control cell enumeration systems is widely recognized (Brockwell and Bottomley, 1995; Tiesongrusmee, 1991).

This study therefore aims to assess the microbial quality of diverse inoculants already available in the markets in order to verify whether they fulfill the claims of the manufacturer and to give an insight to the quality of current available products. This may significantly contribute to the desired impact of increasing crop productivity as effective and good quality products can be promoted while the proliferation of underperforming products can be prevented.

101.2 MATERIALS AND METHODS

101.2.1 Isolation of the Bacteria Contained in the Products

Sixty-five commercial inoculants were ordered from various private companies worldwide (United States, Argentina, United Kingdom, Australia, etc.). The list of the products is presented in Table 101.1.

The inoculants were serially diluted in sterile saline (0.9% (w/v) NaCl) up to 10^{-8} and 0.1 ml aliquot of each dilution was evenly spread on two culture media: yeast extract mannitol agar (YEMA) (0.5 g l^{-1} K_2HPO_4, 0.2 g l^{-1}

Table 101.1 Analyzed products: origin and claimed content

Name of Inoculant	Claimed Microorganisms	Company
Accelerate	*Bacillus polymyxa, Streptomyces* spp.	Nutritech solutions, Australia
B.Sub	*Bacillus subtilis*	Nutritech solutions, Australia
Bac up	*Bacillus subtilis*	Biological Control Products, South Africa
Biofix (16 different products: chick pea, 4 common bean, cowpea, faba bean, french bean, green grams, 3 groundnuts, 2 pigeon pea, soybean, tepary bean)	Rhizobia	Mea Ltd., Kenya
Bio N	*Azotobacter* spp.	Nutritech solutions, Australia
Bioplant	*Bacillus, Clostridium, Achromobacter, Streptomyces, Aerobacter, Nitrobacter, Nitrosomonas.*	Artemis & Angelio Co. Ltd, Thailand
Bioplex	*Azotobacter* spp.	Nutritech solutions, Australia
Chick Pea Nodulator	*Mesorhizobium ciceri*	Becker Underwood, USA
Cowpea peat inoculant	Rhizobia	Becker Underwood, USA
Defender	*Bacillus subtilis*	Biological Control Products, South Africa
Dry bean Nodulator	*Rhizobium leguminosarum* biovar *phaseoli*	Becker Underwood, USA
Excalibur Gold	Natural bacteria for field seed	America's Best Inoculant, USA
Fertosolflo	No information	Soygro (Pty) Ltd., South Africa
Graph-Ex	*Bradyrhizobium japonicum*	America's Best Inoculant, USA
Green gram peat inoculant	Rhizobia	Becker Underwood, USA
Groundnut peat inoculant	Rhizobia	Becker Underwood, USA
Histick NT	*Bradyrhizobium japonicum, Bacillus subtilis*	Becker Underwood, USA
Integral	*Bacillus subtilis* strain MIB600	Becker Underwood, USA
Legumefix (soybean)	*Bradyrhizobium japonicum*	Legume technologies Ltd., UK
Legumefix (common bean)	*Rhizobium* spp.	Legume Technologies Ltd., UK
Leguspirflo	*Azospirillum brasilense -CD*	Soygro (Pty) Ltd., South Africa
Myco Apply MAXX	*Bacillus licheniformis, B. pumilus, B.amyloliquefaciens, B. megaterium*	Mycorrhizal Applications, Inc. USA
Myco Apply Soluble MAXX	*Bacillus licheniformis, B. azotoformans, B. megaterium, B. coagulans, B. pumilus, B. thuringiensis, B. stearothermophilus, Paenibacillus polymyxa, P. durum, P. gordonae, Azotobacter polymyxa, A. chroococcum, Saccharomyces cerevisiae, Pseudomonas aureofaciens, P. florescence*	Mycorrhizal Applications, Inc. USA
Mazospirflo - 2 different types	*Azospirillum brasilense -CD*	Soygro (Pty) Ltd., South Africa
Myco tea	*Azotobacter chroococcum, Bacillus polymyxa*	Nutritech solutions, Australia
Nemablok	No information	Soygro (Pty) Ltd., South Africa
NIB PGPR peat inoculant	*Pseudomonas* sp.	Murdoch University, Australia
Nodulator (faba bean, pea, lentil)	*Rhizobium leguminosarum* biovar *viceae*	Becker Underwood, USA
Nodulator (soybean)	*Bradyrhizobium japonicum*	Becker Underwood, USA
-Nutrilife 4/20	*Bacillus subtilis, Azotobacter vinelandii, A. chroococcum, Pseudomonas fluorescens, P. putida, P. stutzeri, P. cellulose, Bradyrhizobium japonicum, Azospirillum brasilense*	Nutritech solutions, Australia
Peanutflo	*Bradyrhizobium sp.*	Soygro (Pty) Ltd., South Africa
PHC Biopak	*Bacillus azotofixans, B. licheniformis, B. megaterium, B. polymyxa, B. subtilis, B. thuringiensis*	Plant Health Care, Inc. USA
PHC Colonize AG	*Paenibacillus azotofixans, Bacillus licheniformis, B. megaterium, B. polymyxa, B. subtilis, B. thuringiensis*	Plant Health Care, Inc. USA

Table 101.1 (*Continued*)

Name of Inoculant	Claimed Microorganisms	Company
PHC Complete plus	*Bacillus azotofixans, B. licheniformis, B. megaterium, B. polymyxa, B. subtilis, B. thuringiensis, Streptomyces griseoviridis*	Plant Health Care, Inc. USA
Rhizostim	*Azospirillum* sp.	Soygro (Pty) Ltd., South Africa
Rizo-Liq (5 products: chickpea, common bean, green gram, groundnut, soybean)	*Bradyrhizobium* sp. (green gram, ground nut and soybean, Mani 1), *Mesorhizobium ciceri* (chickpea), *Rhizobium* spp. (common bean)	Rizobacter, Argentina
Rizo-Liq Top - 2 different types	*Bradyrhizobium japonicum*	Rizobacter, Argentina
Root plus growth medium	*Paecilomyces lilacinus*	Biological Control Products, South Africa
Soyflo	*Bradyrhizobium japonicum* strain WB74	Soygro (Pty) Ltd., South Africa
Subtilex	*Bacillus subtilis* MBI 600	Becker Underwood, USA
Twin-N	*Azorhizobium, Azoarcus* and *Azospirillum* spp.	Mapleton Int. Australia
Vault LVL	*Bradyrhizobium japonicum*	Becker Underwood, USA
Vault NP	*Bradyrhizobium japonicum*	Becker Underwood, USA

$MgSO_4 \cdot 7H_2O$, 0.1 g l^{-1} NaCl, 1 g l^{-1} yeast extract, 10 g l^{-1} mannitol, 15 g l^{-1} agar) was used to cultivate the rhizobia, while nutrient agar (NA) (1 g l^{-1} meat extract, 2 g l^{-1} yeast extract, 5 g l^{-1} peptone, 5 g l^{-1} NaCl, 15 g l^{-1} agar) was used as a nonselective media for the growth of most other microorganisms.

The YEMA and NA plates were incubated at 28 and 37 °C, respectively, which are the optimal growth temperatures for most of the targeted microorganisms. Plates were observed daily to record colony growth. Colony morphology including shape, outline, texture, size, color, and opacity was noted each day. Different bacterial colonies identified on the same media were selected and purified three times on the same media using a streak plate technique. Pure bacterial cultures were cultivated in liquid media and stored in 20% glycerol at −80 °C for subsequent analyses.

101.2.2 Bacterial DNA Extraction, Amplification, and Sequencing

Total DNA was extracted from purified bacterial cultures following the protocol described by Wilson (Wilson, 1987) with some modifications. Briefly, liquid culture of each strain was grown at the appropriate temperature until it reached its maximal growth. The culture was then centrifuged and the pellet rinsed with TE buffer (10 mM Tris HCl, 1 mM EDTA pH = 8). The pellet was resuspended in 540 µl of 5× TE (50 mM Tris HCl, 5 mM EDTA pH = 8) and incubated for 15 min at 70 °C before being treated with 2 µl of Proteinase K (10 mg ml^{-1}) and 30 µl of 10% (w/v) SDS at 70 °C for 15 min. DNA was extracted with 600 µl of 25 : 24 : 1 (v/v/v) phenol/chloroform/isoamyl alcohol and residual phenol was removed by adding chloroform/isoamyl alcohol. DNA was precipitated overnight in one volume of cold isopropanol at −20 °C. The pellet was cleaned with 70% ethanol, air-dried, and resuspended in 100 µl of sterile micropure water. The samples were treated with 10 µl of 40 µg ml^{-1} RNase and incubated for 30 min at 37 °C to remove any traces of RNA.

A fragment of the 16S rDNA region was amplified using the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Lane, 1991; Turner et al., 1999). The 25 µl reaction volume contained 12.5 µl of Faststart PCR Master Mix (Roche), 8.5 µl of sterile micropure water, 10 pmoles of each primer, and 2 µl of template DNA. Amplification conditions were as follows: predenaturation (94 °C for 5 min); 35 cycles of denaturation (94 °C for 1 min), annealing (55 °C for 1 min), and elongation (72 °C for 2 min); and final elongation (72 °C for 15 min). PCR products were purified with 3M sodium acetate and absolute ethanol and 10 µl at a concentration of 50 ng μl^{-1} was submitted for sequencing at SegoliP Unit (BecA, Nairobi).

101.3 RESULTS AND DISCUSSION

A total of 65 inoculant products were classified in three categories based on their claimed contents. Thirty-nine products out of the 65 tested in this study claimed to contain one or several strains of rhizobia; seven products were supposed to contain free-living N-fixing bacteria (e.g., *Azospirillum*, *Azotobacter*); and other 16 claimed to contain various non-N-fixing PGPR (e.g., PSB, biocontrol agents, etc.) such as *Bacillus* or *Pseudomonas*. Three manufacturers refused to give any information about the contents of their products. The microbial content of these three products was analyzed as for the other products, but their quality couldn't be evaluated.

From the 65 products, a total of 259 strains were isolated, purified, and identified. Several colony types were obtained for most of the products, on both culture media. Ninety-two strains were isolated on YEMA and 167 strains on NA. More than 50% of the inoculants analyzed yielded

Table 101.2 Microbial quality of the inoculants for each type of products

Microorganisms Contained in the Products	Category	Number of Products	(%)	Category	Number of Products	(%)
Rhizobia	Pure	18	46.15	Less strains than expected	0	0
	Intermediate	3	7.69	As many strains as expected	18	46.15
	Contaminated	18	46.15	More strains than expected	21	53.85
	Total	39	100	Total	39	100
Free N fixing	Pure	2	28.57	Less strains than expected	0	0
	Intermediate	2	28.57	As many strains as expected	2	28.57
	Contaminated	3	42.86	More strains than expected	5	71.43
	Total	7	100	Total	7	100
Other PGPR	Pure	3	18.75	Less strains than expected	5	31.25
	Intermediate	10	62.50	As many strains as expected	3	18.75
	Contaminated	3	18.75	More strains than expected	8	50.00
	Total	16	100	Total	16	100

Table 101.3 Microbial quality of the inoculants (regardless of the type of products)

Category	Number of Products	(%)
Pure	23	37.10
Intermediate	15	24.19
Contaminated	24	38.71
Total (classified)	62	100
Not classified	3	–
Total (analyzed)	65	–

more strains than expected suggesting high levels of contamination (Table 101.2). Differences in contamination levels were observed between the N-fixing inoculants and the PGPR inoculants. The N-fixing products generally claimed to include one or two strains only and showed a reasonable number of contaminants. In most cases, inoculants acclaimed to contain one single strain had less than 3 contaminants. N-fixing bacteria and rhizobia in particular are known to require specific conditions for their optimal growth. As a result, the carrier for rhizobial products may be more selective than that of other PGPR strains and discourage the growth of other strains that the inoculant might contain.

The non-N-fixing products (PGPR) usually claimed to contain a large number of strains, up to 15 for some of the analyzed products. The competitive ability of indigenous over inoculant strains has caused some authors to advocate mixed inoculant strain formulations (Kyei-Boahen et al., 2002; Sutherland et al., 2000). Sutherland et al. (2000) suggested that inoculation with a single strain could be risky because the rhizosphere competence of a particular strain to establish an effective symbiosis varies within a wide range of environmental factors and, as a result, one strain may not be effective under all environmental conditions. However, most of the products claiming to have a large number of strains yielded fewer strains than claimed. That could be explained by the difficulty in choosing a carrier suitable for all strains. Ideally, a carrier should provide optimal conditions to allow the bacteria to survive in large numbers during formulation, transport and storage (see also Chapter 97). The optimal conditions might be different for each bacterial strain, and the manufacturers must also ensure that the different strains do not compete with each other (Malusa et al., 2012; Stephens and Rask, 2000). For instance, Kellman (2008) showed that some strains contained in an inoculant were able to produce bacteriocins or antibiotics in order to suppress the growth of "competitors" strains in the mixture.

All the strains isolated from the inoculants were identified after partial sequencing of the 16S rDNA. Results showed that in overall, only 37% of the products contained the expected strain(s) without any contamination. These products can thus be considered as "pure" (Table 101.3). About 24% of all the tested inoculants were classified as "intermediate products" since they contained some or all of the expected strains but with also one or more other strains,

Table 101.4 Pathogenicity and occurrence of the bacterial contaminants isolated from the inoculants

Contaminant	Pathogenicity	References	Occurrence (% of Products)
Agrobacterium sp.	Plant pathogen – some cases of septicemia	Freney et al. (1985); Keane et al. (1970)	3.23
Agrobacterium tumefaciens	Plant pathogen (causes crown gall disease = formation of tumors)	Moore et al. (1997); Smith and Townsend (1907)	9.68
Arthrobacter sp.	Nonpathogenic		4.84
Bacillus amyloliquefaciens	Mostly nonpathogenic (but 1 case of pathogenicity on infants)	Huang et al. (2010)	9.68
Bacillus cereus	Responsible for a minority of foodborne illnesses. Toxin production	Kotiranta et al. (2000); Taylor et al. (2005)	11.29
Bacillus circulans	Mostly nonpathogenic (but 1 case of soft rot on Date Palm)	Leary et al. (1986)	3.23
Bacillus firmus	Mainly not pathogenic (but capable of toxin production)	Taylor et al. (2005)	6.45
Bacillus fumarioli	Nonpathogenic		1.61
Bacillus humi	Nonpathogenic		1.61
Bacillus licheniformis	Toxin production	Salkinoja-Salonen et al. (1999)	4.84
Bacillus massiliensis	Isolated from cerebrospinal fluid	Glazunova et al. (2006)	1.61
Bacillus megaterium	Toxin production	Taylor et al. (2005)	1.61
Bacillus nealsonii	Nonpathogenic		1.61
Bacillus simplex	Toxin production	Taylor et al. (2005)	1.61
Bacillus sp.	Pathogens (for some species)	Sliman et al. (1987)	35.48
Bacillus subtilis	Nonpathogenic		8.06
Bacillus thermoamylovorans	Nonpathogenic	Combet-Blanc et al. (1995)	1.61
Bacillus thuringiensis	Nonpathogenic for human but insect pathogen	Bravo et al. (2011); Raymond et al. (2010)	1.61
Bacillus vallismortis	Nonpathogenic	Roberts et al. (1996)	1.61
Brevibacillus borstelensis	Nonpathogenic	Shida et al. (1996)	3.23
Brevibacillus brevis	Nonpathogenic	Shida et al. (1996)	3.23
Cellulosimicrobium sp.	Rare and opportunistic pathogen	Petkar et al. (2011); Rowlinson et al. (2006)	1.61
Chryseobacterium sp.	Opportunistic human pathogen	Bloch et al., 1997; Padmaja et al. (2006)	1.61
Comamonas testosteroni	Human pathogen	Abraham and Simon (2007)	1.61
Cupriavidus sp.	Opportunistic human pathogen	Langevin et al. (2011)	1.61
Enterobacter pulveris	Nonpathogenic	Stephan et al. (2008)	3.23
Enterobacter sp.	Nosocomial pathogen	Gaston (1988)	3.23
Lysinibacillus fusiformis	Toxin production	Wang et al. (2010)	1.61
Lysinibacillus sphaericus	Opportunistic human pathogen, insect pathogen	Berry (2012)	1.61
Microbacterium hydrocarbonoxydans	Opportunistic human pathogen	Gneiding et al. (2008)	1.61
Microbacterium sp.	Opportunistic human pathogen	Gneiding et al. (2008)	3.23
Moraxella osloensis	Rare and opportunistic pathogen	Tan and Grewal (2001)	1.61
Nocardioides dubius	Nonpathogenic	Yoon et al. (2005)	1.61
Ochrobactrum intermedium	Opportunistic human pathogen	Berg et al. (2005)	3.23
Ochrobactrum sp.	Opportunistic human pathogen	Berg et al. (2005)	4.84
Paenibacillus alvei	Nonpathogenic	Ash et al. (1993)	1.61
Paenibacillus barengoltzii	Nonpathogenic	Osman et al. (2006)	1.61
Paenibacillus lactis	Nonpathogenic	Scheldeman et al. (2004)	3.23
Paenibacillus lautus	Nonpathogenic	Heyndrickx et al. (1996)	1.61
Paenibacillus polymyxa	Nonpathogenic	He et al. (2007)	3.23
Paenibacillus sonchi	Nonpathogenic	Hong et al. (2009)	1.61
Pantoea agglomerans	Human and plant pathogen	Delétoile et al. (2009)	1.61
Pseudomonas fluorescens	Potential pathogen	Picot et al. (2001)	3.23
Pseudomonas sp.	Pathogens (for some species)	Stead (1992)	3.23

Table 101.4 (*Continued*)

Contaminant	Pathogenicity	References	Occurrence (% of Products)
Pseudoxanthomonas sp.	Nonpathogenic	Thierry et al. (2004)	3.23
Serratia marcescens	Human pathogen	Kurz et al. (2003)	1.61
Solibacillus silvestris	Non pathogenic	Morohoshi et al. (2011)	1.61
Staphylococcus pasteuri	Potential pathogen. Toxin production	Chesneau et al. (1993)	1.61
Stenotrophomonas maltophilia	Human pathogen	Looney et al. (2009)	4.84
Stenotrophomonas sp.	Opportunistic human pathogen	Berg et al. (2005)	4.84
Total (all products)			53.23
Total (contaminated products)			84.62

considered as contaminants. Finally, almost 40% of the inoculants contained none of the claimed strains by the manufacturers but only contaminations and were referred to as "contaminated products" (Table 101.3). Some of the isolated strains which were not identified by the manufacturers may be shown to also have plant growth-promoting activities. However, for the purposes of this study, if not claimed by the manufacturers, they must be referred to as "contaminants."

Inoculants containing rhizobia were generally of better quality than the rest of the inoculants since almost half of the rhizobial inoculants (46%) tested in this study were classified as pure products, while a further 46% contained none of the expected strain(s) but only contaminants (Table 101.2). It is important to note that 15 out of the 18 rhizobial inoculants classified as "contaminated" were sold by the same company. Indeed, only one product (out of 16) sold by this company actually contained the strain of rhizobia it was claimed to contain (plus some contaminants). It is likely that inappropriate sterilization of the carrier, impurity of the strains before inoculation or contamination of the packaging material may be responsible for the high levels of contamination of these products.

Inoculants containing free-living N-fixing bacteria were generally of poor quality: 43% of them were classified as contaminated products and only 28% were pure. Similar results were obtained with the products claiming to contain non-N-fixing PGPR: only 19% of products were found to be pure, while a majority of the products (62 and 19%, respectively) belonged to the intermediate or contaminated categories (Table 101.2). Moreover, in most cases, when a company did not give the details of the product's microbial content, the same product ended up containing a mix of several strains.

Overall, these results show a remarkably high level of contamination across the range of products. The bulk of "contaminants" found were *Bacillus* sp., but other genera were found such as *Brevibacillus*, *Pseudomonas*, *Moraxella*, or *Staphylococcus*. The list of the contaminants found in the products is given in Table 101.4.

A similar study by Olsen et al. (Olsen et al., 1996) reported on the contaminants found in 60 samples of commercial inoculants: 25% of the inoculants contained opportunistic human pathogens at high levels. Many of these pathogenic strains were also found in our study (such as *Pseudomonas* sp., *Agrobacterium sp.*, *Enterobacter sp.*, *Staphylococcus sp.*), occurring in more than 53% of the products tested (all categories included) and almost 85% of the contaminated products (Table 101.4). Although some of the strains are known to be very rarely pathogenic, our results show that a significant portion of the inoculants are potentially dangerous for humans, plants, or the environment, and this is largely superior to the proportions obtained by Olsen et al. (1996). Other studies have reported bacterial contamination including human pathogens such as *Acinetobacter, Flavobacterium, Alcaligenes, Pseudomonas, Moraxella*, and *Enterobacter* or bacteria inhibiting the growth of rhizobia (Azpilicueta et al., 1996; Gomez et al., 1997; Olsen et al., 1994a). The presence of bacteria which may represent a risk for humans, plants, and the environment poses issues beyond just being contaminants in the inoculant products and should not be ignored (Bashan, 1998; Catroux et al., 2001).

The issue of the quality of inoculants has been raised for many years. Several authors described as a paradox the fact that despite almost 100 years of research and experience, a large portion of the inoculants produced in the world today are still of poor and sometimes extremely poor quality because quality controls are not systematically practiced (Brockwell and Bottomley, 1995; Catroux et al., 2001; Somasegaran, 1991). There have been examples of rhizobial inoculants sold on the markets that did not contain any rhizobia at all (Olsen et al., 1996). In many countries, there is currently neither a system in place nor a set of common international standards to evaluate and ascertain the quality of the products that reach the end user (Olsen et al., 1994b).

To counter this problem, some countries have developed an appropriate legislation supporting systems to control the quality of the products. For instance, in Canada, the quality of all inoculants produced is controlled and an annual

report is prepared (Anonymous, 1997; Catroux et al., 2001). In France, the requirements for inoculant quality are some of the most stringent, with no detectable contaminants admitted (Catroux, 1991). In other countries (e.g., Australia, Thailand, New Zealand), the quality control is voluntary on the part of the inoculant manufacturers (Catroux et al., 2001; Herridge et al., 2002). On the other hand, many countries have not set up or implemented such standards (Brenner, 1996). As a consequence, almost anyone can sell an inoculant without going through the process of quality check. The results obtained in the present study are in accordance with these observations as a majority of the inoculants were not of good quality. This confirms the importance of an effective, regulatory quality control program to ensure a successful production and use of inoculants (see also Chapter 97).

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Chapter 102

Developed Fungal-Bacterial Biofilms Having Nitrogen Fixers: Universal Biofertilizers for Legumes and Non-Legumes

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102.1 INTRODUCTION

A biofertilizer is an agricultural input, which contains living microorganisms exerting direct or indirect beneficial effects on plant growth and productivity through various mechanisms (Bohlool et al., 1992). It is also a supplement but a sustainable source for replenishing depleted soil microbial community by increasing biodiversity (Seneviratne and Kulasooriya, 2013). It is reported that genera of *Rhizobium, Bradyrhizobium, Azospirillum, Azotobacter, Burkholderia, Enterobacter, Klebsiella, Pseudomonas, Azoarcus, Gluconacetobacter, Herbaspirillum*, and many other rhizobacterial genera enhance the plant growth via supplying essential nutrients and/or providing plant growth-promoting substances (Francesco and Jumpponen, 2006). In general, most of the biofertilizers consist of one or many of the above mentioned bacterial types as "active ingredients" (see Chapter 101).

Many studies have revealed that widely used plant growth-promoting rhizobacteria show specific interactions with their host plants (Saharan and Nehra, 2011; Francesco and Jumpponen, 2006). A typical example is the symbiosis established between bacteria of the Rhizobiaceae and the plant family Leguminosae (Rhijn and

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Vanderleyden, 1995). Similar interactions are documented within *Azospirillum* (see Chapter 90), *Herbaspirillum* (see Chapter 93), and cereal crops (Baldani, and Dobereiner, 1980). Azotobacter is another example that shows species-based-specific plant interactions (Tilak et al., 2009). Based on those interactions, specific biofertilizers have been formulated for specific crops. Thus, most of the currently available biofertilizers are suitable for one particular or a narrow range of crops. Due to this specificity, plant-specific biofertilizer production creates problems at the industrial level, especially large-scale biofertilizer production. If a few types of biofertilizers could be formulated to a wide range of crops that would certainly provide benefits to commercial biofertilizer industry. Further, the crop response to conventional biofertilizers is also a concern of farmers, due to marginal improvement of yields.

As a recent development in microbiology, surface-attached microbial communities or biofilms are being studied for many biotechnological applications. Biofilms are often complex communities of multiple microbial species that remain attached to surfaces (Seneviratne et al., 2009). They consist of microbial cells and sticky extracellular polymeric substances (EPS; see Chapter 36) which provide structure and protection to the community (Flemming and Wingender, 2010). In this field of research, bacteria in the fungal surface-attached biofilm mode are called fungal-bacterial biofilms [FBBs], which can be developed *in vitro* from microbial monocultures (Seneviratne et al., 2008). These developed biofilms can be applied for various agricultural and biotechnological purposes. As a novel advancement in biofertilizer research, FBBs have been developed and are being tested as biofertilizers, which are now known as biofilmed biofertilizers (BFBFs). It has been shown that plant-associated nitrogenase activity, rhizoremediation, plant and soil carbon sequestration, and plant growth-promoting activities are enhanced by FBBs (Seneviratne et al., 2009). Also, these well-developed biofilms can enhance microbial effectiveness compared to monocultures. It was observed that microbial interactions in the FBBs fixed N_2 biologically, as revealed by nitrogenase activity and N-accumulation, which was not observed when a rhizobial strain was used alone as a monoculture (Jayasinghearachchi and Seneviratne, 2004). Under field conditions, the FBBs have saved 50% of chemical fertilizer use in crop production, with other crop and soil benefits, but without lowering yields (Seneviratne et al., 2011). As such, those observations have made the FBBs more attractive to farmers than the conventional biofertilizers.

The biofilm structures are physiologically and anatomically exclusive and their dynamism and processes are quite different from monocultures (Hancock, 2001). In most biofilms, the microorganisms account for less than 10% of the dry mass, whereas the matrix can account for over 90%. The matrix is the extracellular material, which consists of a conglomeration of different types of biopolymers such as EPS (Flemming and Wingender, 2010; see Chapter 36). As a result, genomic functions and regulations are reasonably diverse in the FBBs. This may cause varied production of different biochemical molecules in the FBBs (Herath et al., 2013) and may lead to break plant-microbe specificity, thus facilitating universal behavior of biofertilizers.

Therefore, *in vitro* development of FBBs with nitrogen fixers and their promotion are essential steps to overcome low acceptance of the biofertilizers by farmers, as well as in the industry. This chapter highlights recent advances in the application of the FBBs, focusing on the potential effects of them on leguminous as well as non-leguminous crops.

102.2 METHODOLOGY

Experiments were conducted at the Institute of Fundamental Studies [IFS], Kandy, Sri Lanka [midcountry wet zone [WM3], 510 m amsl; mean annual temperature, 25.5 °C]. Soil type in the site was Red-Yellow Podzolic with the texture of clay loam, pH 4.1, 1.45% total C, and 0.08% total N. Common bean [*Phaseolus vulgaris L.*], tomato [*Solanum lycopersicum L.*], capsicum [*Capsicum annuum L.*], cabbage [*Brassica oleracea* var. capitata], and rice [*Oryza sativa L.*] crops were employed to evaluate the universality of developed microbial biofilms.

The FBBs used in this study were developed mainly by coculturing of nitrogen-fixing bacteria and a saprophytic fungus (Seneviratne et al., 2011). When they were cocultured *in vitro*, the bacteria attached and colonized on fungal mycelia to form the FBB. The bacterial strains used in this study were *Acetobacter, Azotobacter, Bradyrhizobium*, and *Rhizobium*. *Acremonium* sp. was used as the fungal strain. During bacterial biofilm development process, their maturity at different stages was observed microscopically. Simultaneously, nitrogen-fixing ability was measured using acetylene reduction assay [ARA; Zuberer and Silver, 1978]. To this end, gas samples collected from the headspace of the vial [0.5 ml] were injected into a gas chromatograph [Shimadzu GC 9AM, with a hydrogen flame equipped with flame ionization detector [FID]], a Carboxen™-1010 PLOT capillary column, and helium [99.99%] as the carrier gas at a flow rate of 0.625 ml s^{-1}.

To evaluate the effect of the FBB, three treatments were applied to the four non-legume crops: (i) half of the recommended fertilizer (T1), (ii) developed FBB + half of the recommended fertilizer (T2), and (iii) control (no fertilizer or biofilm). For common bean, treatments were; biofilmed biofertilizers (BFBFs), 100% recommended chemical fertilizers, and no fertilizer control. Each treatment had five replicates, and the experiment was arranged according to completely randomized design CRD.

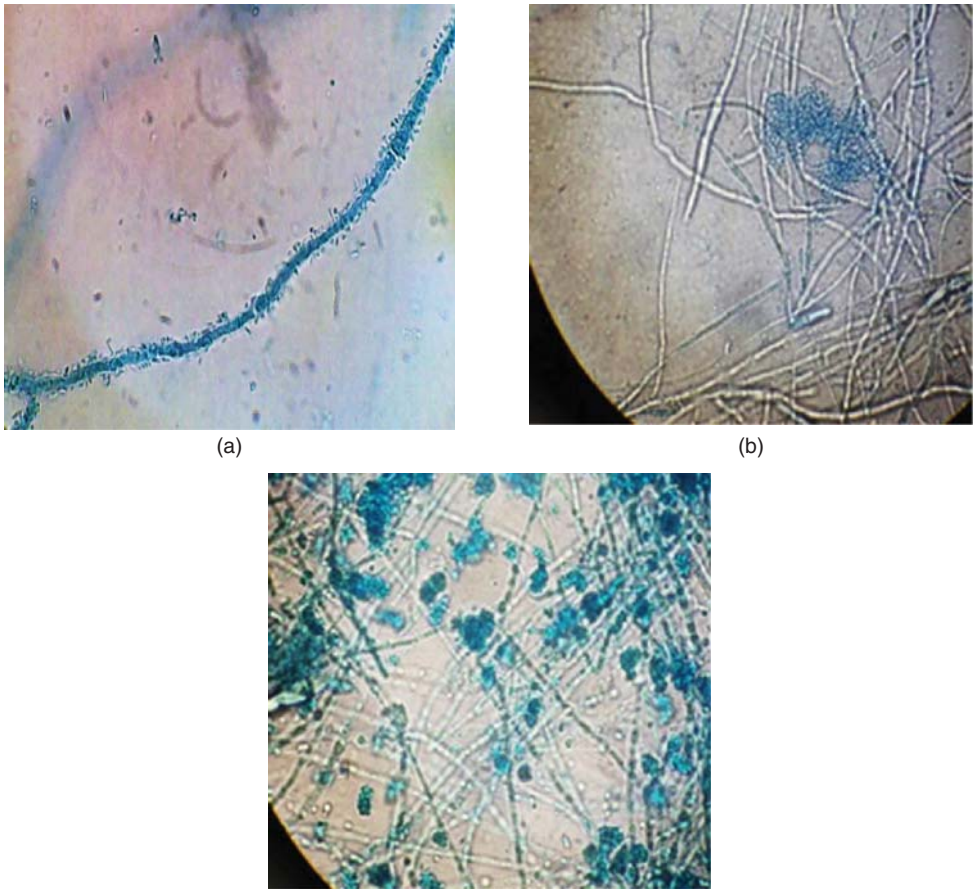

Figure 102.1 *Acremonium* sp. mycelial colonization by N_2-fixing bacteria (*Acetobacter*, *Azotobacter*, *Bradyrhizobium*, and *Rhizobium*) forming fungal-bacterial biofilms, when developed under *in vitro* conditions. (a) Initial colonization of bacteria of the fungal mycelium, (b) a cell cluster formed by multiplication of the colonized bacteria, and (c) matured cell clusters on the mycelium. Darkness is due to cotton blue stain absorbed by exopolysaccharides produced by the biofilms. Magnification, × 400.

Twelve weeks after transplanting, five representative plants were randomly uprooted from each plot. Representative root samples were subjected to ARA. In the case of common bean, roots, and nodules were separately subjected to ARA. After that, all shoots and clean roots were oven dried at 65 °C for total dry weight measurements.

The effects of the treatments were analyzed by ANOVA at 5% probability level, and means were separated by using Tukey's HSD test. SAS® 9.1.3 software was used for all statistical analyses.

102.3 RESULTS

Figure 102.1 shows three stages of FBB development under *in vitro* conditions over time. Initially, *Acremonium* sp. mycelium was colonized by cocultured bacterial species of *Acetobacter, Azotobacter, Bradyrhizobium*, and *Rhizobium* (Fig. 102.1a). Then, cell clusters were formed through multiplication of the colonized bacteria (Fig. 102.1b). At third stage, maturation of the biofilms occurred, as reflected by dark cell clusters on the mycelium, which were stained by cotton blue stain that was absorbed by exopolysaccharides (EPS) produced by the biofilms (Fig. 102.1c). Simultaneously, nitrogenase activity of the developed FBB was measured and found to increase with the maturation stage of FBBs (Fig. 102.2).

Twelve weeks after transplanting, shoot and root dry weights of tomato, capsicum, cabbage, and rice plants treated with the FBB together with half of the recommended fertilizers (T2) were found to be significantly higher than that of 50% recommended chemical fertilizer-added plants (T1) (Fig. 102.3). T1 treatment showed an inconsistent plant growth. Similarly, total dry weight of biofilm-treated common bean plants was significantly higher than that of the chemical fertilizer treated or no fertilized plants (Fig. 102.4).

Root, shoot, and pod dry weights also followed the same trend. However, nodule dry weight did not show significant differences among the treatments [$p > 0.05$].

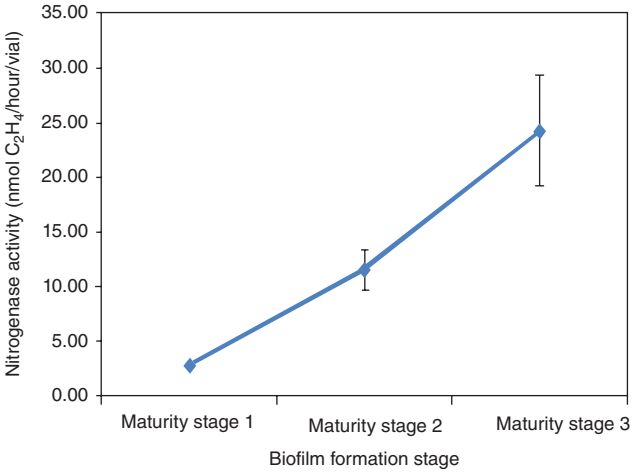

Figure 102.2 Change in nitrogenase activity during fungal mycelial colonization of bacteria and biofilm formation at three stages of maturation, described in Figure 102.1.

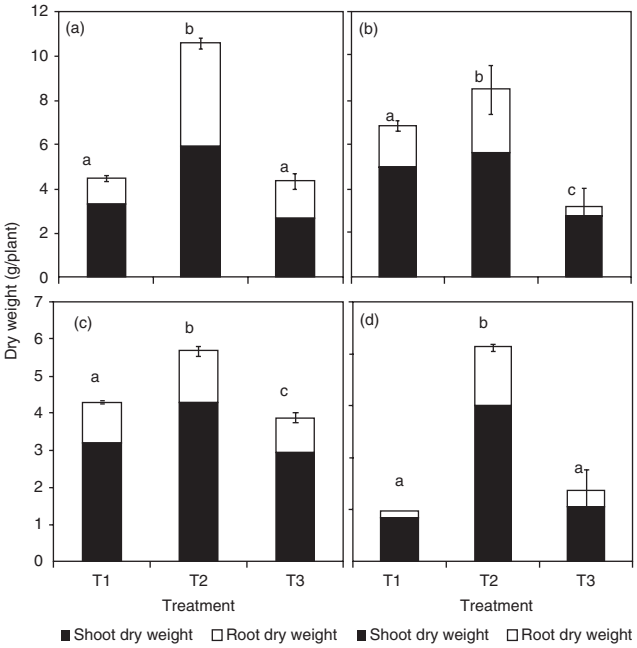

Figure 102.3 Shoot and root dry weights of (a) tomato, (b) capsicum, (c) cabbage, and (d) rice, with half of the recommended fertilizers (T1), FBB + half of the recommended fertilizers (T2), and no fertilizer (T3). Columns sharing a same letter do not significantly differ at 5% probability level. Vertical bars show standard errors.

Nodules of the biofilm-treated bean plants showed about sixfold significant increase in nitrogenase activity compared to 100% recommended chemical fertilizer-added treatment (Fig. 102.5). The biofilm-treated roots without nodules also positively responded to the assay, showing about fourfold significant increase compared to the full chemical fertilizer treatment. Microscopic observations revealed that the root samples that responded positively to nitrogenase activity contained root surface- and root hair-attached biofilm structures (Fig. 102.6). Similar biofilm structures attached to the roots were also observed in capsicum, cabbage, and rice treated with the biofilms.

102.4 DISCUSSION

Biofilm formation generally follows a sequence of steps (Tenke et al., 2004). Initially, planktonic bacteria are deposited on biotic or abiotic surfaces, and subsequently, the cells adhere and anchor to the surface by EPS. Thereafter,

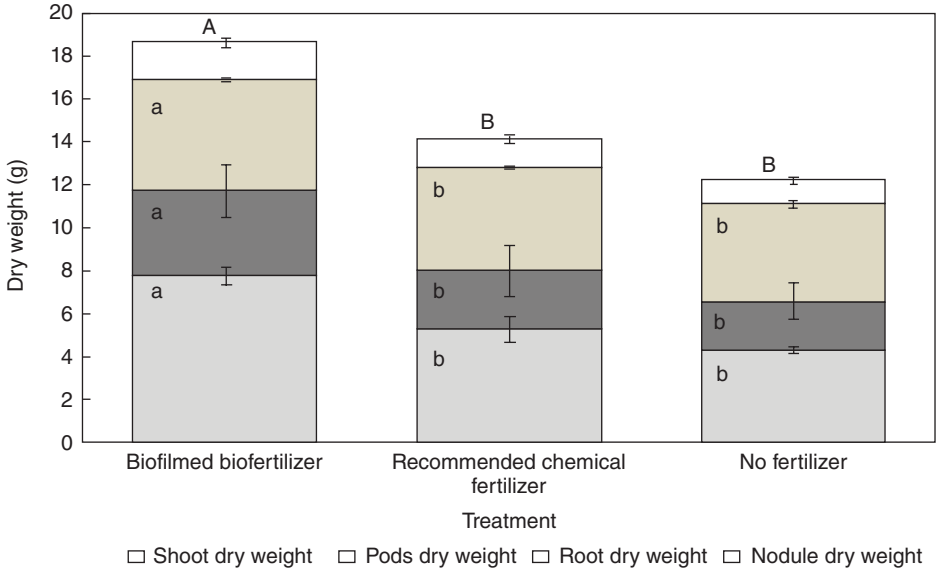

Figure 102.4 Shoot, pod, root, and nodule dry weights of common bean (*Phaseolus vulgaris* L.) treated with the FBB, recommended chemical fertilizer dose, and no fertilizer. Different letters across the columns show significant differences at 5% probability level, whereas the absence of letters indicates absence of significant differences at the same probability level. Vertical bars show standard errors.

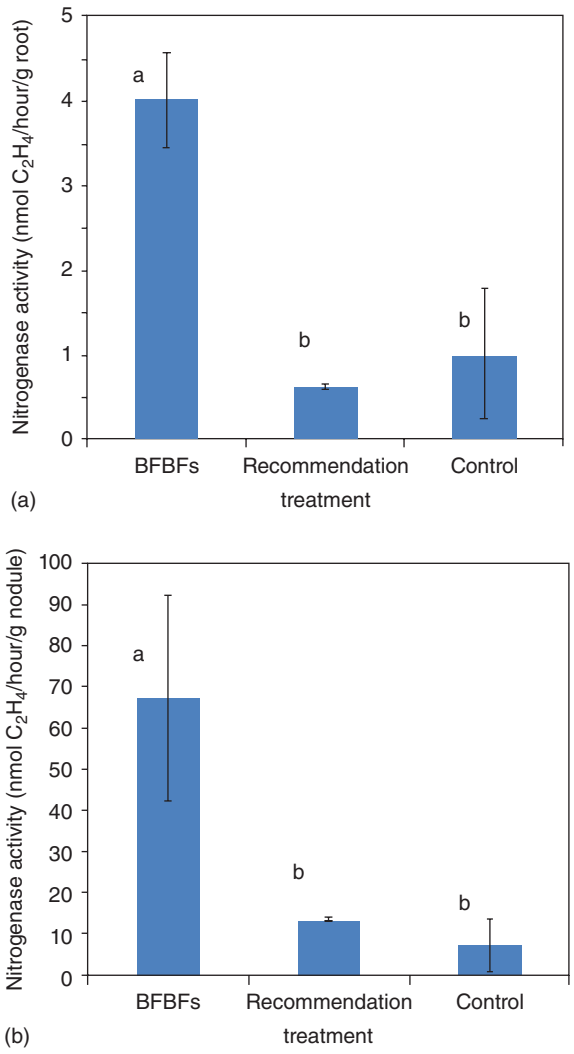

Figure 102.5 (a) Nitrogenase activity of common bean roots (without nodules), and (b) nitrogenase activity of common bean root nodules of the treatments. Columns sharing a same letter do not significantly differ at 5% probability level. Vertical bars show standard errors.

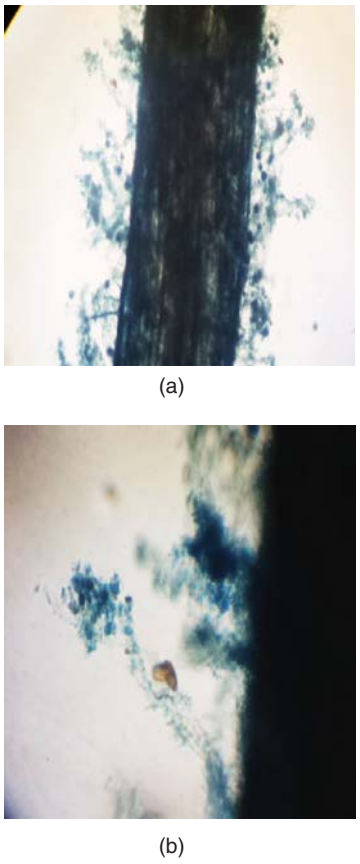

Figure 102.6 (a) and (b) Tomato root surface and root hair colonized by developed microbial biofilms. Stain, lactophenol cotton blue. Magnification, × 400.

the biofilm growth and multiplication occur. In the present study, *Acremonium* sp. mycelium was colonized by nitrogen-fixing bacteria *Rhizobium* sp., *Bradyrhizobium* sp., *Acetobacter* sp., and *Azotobacter* sp., which led to the formation of FBB (Seneviratne and Jayasinghearachchi, 2005). With maturation of biofilms, EPS are accumulated as the matrix (Flemming and Wingender, 2010). Increased nitrogenase activity with maturity could be due to the presence of a higher number of nitrogen fixers in the biofilm or enhancement of nitrogen fixation in the matured community. This needs further investigations.

The FBBs are capable of attaching to plant root system, creating root-biofilm associations, which benefit both plants and biofilms (Seneviratne et al., 2009). The root-attached biofilms with nitrogen fixers have been reported to behave as nodule-like structures or "pseudo nodules", which can fix nitrogen biologically (Jayasinghearachchi and Seneviratne, 2004). These special structures on the root system support non-leguminous plants to supplement their nitrogen requirement more efficiently. It is speculated that the pseudonodules with nitrogen fixers are capable of transferring fixed nitrogen to the root after death and decomposition of the biofilm structures. In return, the root may supply carbon sources to the biofilm (Seneviratne et al., 2009). Release of organic acids by the biofilms helps to increase mineralization of soil nutrients in the rhizosphere (Seneviratne and Jayasinghearachchi, 2005). Increased production of plant growth-promoting hormones like indole acetic acid [IAA] by biofilms helps to enhance plant growth (Bandara et al., 2006). In addition, the FBBs are important in replenishing beneficial microbial communities in depleted agricultural soils due to heavy use of chemical inputs and intensive cropping (Seneviratne et al., 2009). All the aforementioned factors involving the FBBs contribute to increased plant growth, compared to conventional chemical fertilizer application.

Application of moderate levels of inorganic fertilizers helps to increase the composition and biomass of microbes

in the FBBs treated soil. As a result of this, biofilm communities with high cell densities are colonizing root system, which can increase plant growth (Seneviratne, 2009). In the present study, possibility of using only 50% of the chemical fertilizers for the entire crop of such nonlegumes would be a huge economic gain in terms of fertilizer saving.

As observed in the present study, the developed microbial biofilms are also capable of colonizing root system of legume plants enhancing nitrogenase activity of the rhizosphere. As a result, vertical roots can be colonized by the biofilm, which are then able to form the pseudonodules fixing N_2, in addition to proper root nodules. This extra arrangement supplies more nitrogen to the plant, thus resulting in elevated crop growth. It has been also shown that the inoculation of FBBs helps to maintain a higher cell density of rhizobia on the root system than the inoculation of rhizobial monocultures (Seneviratne and Jayasinghearachchi, 2005). Biofilm-based inoculant delivering method may also have a significant effect on common bean nodule production, because biofilm structure provides extra protection and a favorable microenvironment for rhizobia. The root-biofilm association also enhances the legume plant nutrient absorbance from the surrounding soil (Seneviratne et al., 2009). Furthermore, the established microbial communities on the root system may protect the plant from adverse environmental conditions and pathogenic infections (Seneviratne et al., 2010). Importantly, developed microbial biofilms may enhance cell signalling between legume plants and rhizobia, ultimately leading to establish effective symbiosis between them. As a result of all those positive impacts created by the FBBs, common bean showed significantly high plant growth.

102.5 CONCLUSIONS

This study showed that the FBB developed with nitrogen fixers enhanced the plant growth of legumes as well as nonlegumes. Thus, crop specificity of conventional biofertilizers, one constraint in popularizing biofertilizers, can be overcome by using the FBB-based biofertilizers. Further, this eco-friendly and economical biofertilizer technology, due to its reduced use of chemical fertilizers could lead to a sustainable agriculture without hampering yields. Nitrogen-fixing pseudonodules developed on all plant roots may contribute to an increased N budget in the field. However, further laboratory and field studies are required to understand the complete mechanisms and to realize the full potential of the FBBs.

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Chapter 103

Phenotypic Variation in *Azospirillum* spp. and Other Root-Associated Bacteria

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103.1 INTRODUCTION

Phenotypic variation (Pv) is used by several bacterial species to generate intrapopulation diversity that increases bacterial fitness and is important in niche adaptation or to escape host defenses (in the case of pathogens). Pv or phase variation allows that the expression of a given phenotype is either ON or OFF; these events are usually reversible but may be irreversible and result from genetic or epigenetic alterations at specific loci (van der Woude and Baumler, 2004; Wisniewski-Dyé and Vial, 2008). In contrast to spontaneous mutations, occurring at a frequency of approximately 10^{-8} to 10^{-6} mutations per growing cell per generation, Pv occurs at frequencies higher than 10^{-5} events/switches per cell per generation and always affects the same phenotype(s). Pv is extensively studied in animal/human pathogens but has also been reported for plant pathogens and plant beneficial bacteria. In the case of the latter, various phenotypes, such as motility, aggregation, pigmentation, synthesis of antifungal metabolites, and metabolic properties, appear to be regulated through Pv.

In this review, the literature about the occurrence of Pv in different species and strains of the plant growth-promoting diazotrophic genus *Azospirillum* (see Chapter 90) is summarized and complemented with examples of other root-associated and plant growth-promoting bacteria.

103.2 PHENOTYPIC VARIATION IN *Azospirillum* UNDER NORMAL GROWTH CONDITIONS

Azospirillum lipoferum 4B, a strain isolated from a rice rhizosphere in France (Thomas-Bauzon et al., 1982), was reported to generate under normal growth conditions a stable phase variant named $4V_I$ at high frequencies [10^{-4} to 10^{-3} per cell per generation] (Alexandre and Bally, 1999). Variant colonies were distinguishable from wild-type colonies by the differential absorption of dyes (such as bromothymol blue)

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incorporated into the growth medium: whereas wild-type colonies were blue and opaque, variant colonies appeared nearly white and translucent and usually smaller in diameter (Vial et al., 2004). Under fully aerated conditions at 28 °C, variant cells have been detected only when the parental strain entered the exponential growth phase and their number increased proportionally to the total number of viable cells and reached a maximum of about 2% at the end of the exponential phase (Alexandre and Bally, 1999).

The $4V_I$ variant exhibits pleiotropic modifications compared to the wild-ype. First, it gained the ability to assimilate certain sugars (xylose, galactose, α-methyl-mannoside, 5-ketogluconate) but lost the ability to assimilate others (*N*-acetylglucosamine, tagatose, esculin); fermentation of sorbitol, mannitol, and glucose was also abolished (Alexandre and Bally, 1999). Second, the $4V_I$ variant was no longer able to reduce triphenyl tetrazolium chloride (Alexandre and Bally, 1999). Third, the $4V_I$ variant was unable to swim as it lacked a polar flagellum due to a defect in flagellin synthesis, but it constitutively expressed mechanosensing lateral flagella (Alexandre and Bally, 1999; Alexandre et al., 1999b). Fourth, the $4V_I$ variant could no longer reduce nitrous oxide and deaminate 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of plant ethylene (Alexandre, 1998; Prigent-Combaret et al., 2008).

From the $4V_I$ variant that was unable to revert to the parental phenotype, a second atypical stable form, named variant $4V_{II}$, appeared under very specific conditions, that is, growth at extremely low oxygen concentrations. This new variant displayed laccase activity and the ability to produce melanin and was also unable to revert to the parental phenotype (Alexandre and Bally, 1999). Laccases or laccase-like multicopper oxidases (EC 1.10.3.2) catalyze the oxidation of various substrates, such as phenols, diamines, and metals, coupled with the reduction of molecular oxygen to water. When oxidizing aromatic substrates, laccases generate reactive species, such as semiquinones and quinones that are powerful inhibitors of the electron transport system in bacteria (Imlay and Fridovich, 1992).

The electron transport systems of the laccase-positive $4V_{II}$ variant and its parental laccase-negative forms have been compared. During exponential (but not stationary) growth under fully aerobic (but not under microaerobic) conditions, the laccase-positive variant lost a respiratory branch that is terminated in a cytochrome *c* oxidase of the aa3 type (Alexandre et al., 1999a); this observation was most likely due to a defect in the biosynthesis of a heme component essential for the oxidase. Moreover, the $4V_{II}$ variant was significantly less sensitive to the inhibitory action of quinone analogs and fully resistant to inhibitors of the bc1 complex, apparently due to the rearrangements of its respiratory system. Thus, loss of the cytochrome *c* oxidase-containing branch in the variant might be an adaptive strategy to the presence of intracellular oxidized quinones, the products of laccase activity (Alexandre et al., 1999b).

The occurrence of Pv under normal growth conditions has been investigated in a collection of *Azospirillum* strains belonging to various species [*amazonense*, *doebereinera*e, *halopraeferens*, *irakense*, and *lipoferum*]. Five strains out of 27 generated variant colonies exhibiting the same phenotypes as the $4V_I$ variant: they could no longer fix bromothymol blue and reduce triphenyl tetrazolium and were nonmotile (Vial et al., 2006). Interestingly, the stabilities of the variants differed: whereas *A. brasilense* WN1, a strain isolated from wheat in Pakistan, engendered a stable phase variant, subculturing variant colonies from *A. brasilense* Wb1 (wheat, Pakistan) and *A. irakense* KBC1 (rice, Iraq) produced a mixture of wild type and variant colonies (at a ratio of about 10 : 1), suggesting that a reversion event had occurred. For strains *A. brasilense* PH1 (rice, France) and *A. lipoferum* MRB16 (rice, Bangladesh), subculturing of variants yielded almost only wild-type colonies, and thus, variants from these strains were considered highly unstable (Vial et al., 2006). The ability to generate variants could not be correlated to a particular *Azospirillum* species or to a specific plant host, and hence, the adaptive significance of Pv in *Azospirillum* remains to be established.

In a recent study, exopolysaccharide overproducing variants of *A. brasilense* Sp7 were examined in further detail (Table 103.1) (Volfson et al., 2013).

Variant colonies with a much increased mucoid morphology occurred spontaneously with a frequency of about 1 in 5000 cells per generation on fructose-based mineral medium. The variants produced exceedingly mucoid colonies [Figure 103.1] that contained about 8 times more exopolysaccharides.

In addition, the exopolysaccharides of the variants were also different in the relative composition of monosaccharides and showed improved tolerance to heat [55 °C] and UV exposure (Volfson et al., 2013).

103.3 PHENOTYPIC VARIATION IN *Azospirillum* UNDER STARVATION AND STRESS CONDITIONS

During exposure of *A. brasilense* Sp7 to NaCl stress, colonies with resistance toward 3,4-dehydroproline (DHP), an antimetabolite of the osmoprotectant proline (Csonka, 1981), could be isolated. In the presence of 80 μg ml^{-1} DHP and salt stress (0.5M NaCl) in mineral medium, DHP-resistant mutants appeared at a frequency of 10^{-1} per generation (Hartmann et al., 1992). These DHP-resistant isolates were then stable in the absence of salt stress and retained their osmotic stress tolerance. A similar high frequent selection of isolates with the ability to grow at severely iron-limiting conditions could be achieved using the

Table 103.1 Exopolysaccharide (EPS) Production, Cell Aggregation, and Partial EPS Monosaccharide Composition of *Azospirillum brasilense* Sp7 and Three of Its Phenotypic Variants

Strain	EPS/Cell Dry Weight* (g g^{-1})	Cell Aggregation (%)*	Monosaccharide Composition of EPS (%)†			
			Glucose	Galactose	Rhamnose	Mannose
Sp7-parental strain	0.20 b	5.0 c	12.7	24.1	17.6	25.5
Variant phv1	1.50 a	18.0 b	57.9	1.5	32.1	8.4
Variant phv2	1.60 a	18.5 b	32.7	56.2	3.5	7.4
Variant phv3	1.60 a	27.0 a	33.1	44.2	9.9	6.1

*Values represent averages from three independent experiments, each with five replicates for each parameter. Different letters in each column indicate significant differences ($p = 0.05$).

†EPS were purified after 72 h of growth in D-fructose minimal medium. Results represent average ± standard deviation (SD) of the relative presence of each sugar (percentage) from two independent experiments.

(Modified from Volfson et al., 2013).

artificial iron chelator dipyridyl and the fungal siderophores ferrichrysin and coprogen (siderophores which cannot be used by the *A. brasilense* Sp7 wildtype for iron acquisition) (Hartmann, 1988). It has been proven that these isolates were no contaminants, because they showed identical restriction patterns as the wildtype in restriction analysis/pulsed-field gel electrophoresis (Hartmann et al., 1992). Although no detailed studies of genetic alterations were reported on these isolates, they can be considered as phenotypic variants in the light of the present knowledge. These observations indicated very early that there is a potential of considerable phenotypic plasticity in *A. brasilense* Sp7, which may be used applying specific selection pressure conditions to achieve more stress tolerant strains/variants.

More recently, the phenomenon of Pv was studied in more detail in spontaneous aggregation mutants of *A. brasilense* Sp7. This mutant, Sp7-, devoid of aggregation properties, was exposed to carbon starvation for 12 days. Colonies differing in pigmentation (pig), cell aggregation (agg) and EPS overproduction (EPS^{op}) were examined, as these phenotypes have been often associated with increased survival to different stresses in *A. brasilense* (Hartmann and Hurek, 1988; Hartmann et al., 1992; Fibach-Paldi et al., 2012; Lerner et al., 2010). Three different phenotypes were repeatedly observed, although with no indication of frequencies: agg-pig+ (variant $Sp7_2$), agg+pig- (variant $Sp7_3$), and agg-pig+EPS^{op} (variant $Sp7_E$). No differences were observed between LPS patterns of strains Sp7 and Sp7- and the variants $Sp7_2$ and $Sp7_3$; as for variant $Sp7_E$, it lacked the typical high-molecular-weight LPS commonly observed in LPS profiles of *A. brasilense* strains and showed instead modified forms of LPS with lower molecular weights (Lerner et al., 2010). Variant $Sp7_E$ showed significantly higher EPS concentrations (~threefold increase) than the parental strain and the other variants. All variants exhibited modifications in the content of monosaccharide composition of the EPS, the most dramatic changes being observed for $Sp7_E$. Variant $Sp7_E$ formed significantly more biofilm on glass than the parental strain, notably in high C/N ratio. As LPS and EPS contribute to protection against different stresses, variant $Sp7_E$ was further assessed for its ability to respond to several stresses. Variant $Sp7_E$ showed higher survival than the parental strain following exposure to heat (55 °C), osmotic pressure, and desiccation but higher sensitivity to UV radiation and hydrogen peroxide (Lerner et al., 2010). Differences in outer membrane protein (OMP) patterns were noticed between variant $Sp7_E$ and the parental strain Sp7-; interestingly, the OMP pattern of variant $Sp7_E$ displayed similarity with that of a Sp7 mutant altered in LPS biosynthesis (mutated in *noeJ* encoding mannose-6-phosphate isomerase) (Lerner et al., 2009). One can anticipate that such stress-induced events might occur in other *Azospirillum* strains too.

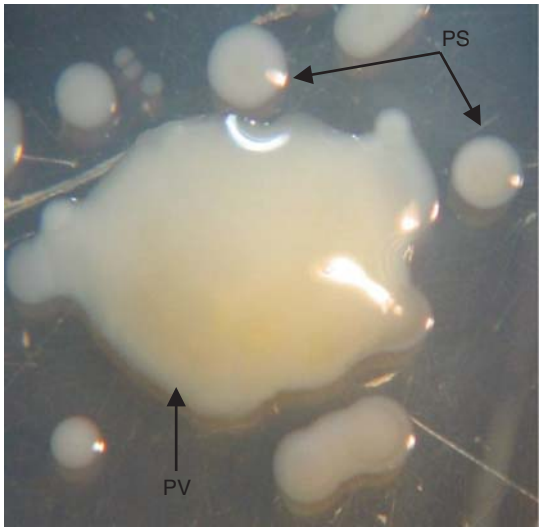

Figure 103.1 Phenotypic variant of *A. brasilense* Sp7. The parental strain (PS) forms a slightly mucoid colony, while isolated phenotypic variants (Pv) generally form exceedingly mucoid colonies as compared with the PS.

103.4 GENOMIC REARRANGEMENTS ASSOCIATED WITH PHENOTYPIC VARIATION IN *Azospirillum*

As Pv is often associated with genomic DNA rearrangements (van der Woude and Baumler, 2004; Wisniewski-Dyé and Vial, 2008), the occurrence of such rearrangements has been investigated in variants obtained from *Azospirillum* strains. Pulsed-field gel electrophoresis (PFGE) following restriction with different enzymes did not reveal any differences between *A. brasilense* Sp7- and its variants (Lerner et al., 2010). However, repetitive PCR (rep-PCR; Rademaker et al., 2000) did reveal differences between wild-type Sp7, Sp7-, and variants obtained from Sp7-. The three rep-PCR techniques (ERIC-, BOX-, and REP-PCR) employed clearly discriminated between strains/variants differing in their aggregation ability but not in pigmentation, suggesting that loss of aggregation involves DNA rearrangements. Variant $Sp7_3$ (agg+pig-) showed identical rep-PCR patterns than that of wild-type Sp7, suggesting that this variant could be a spontaneous revertant of Sp7 (agg-pig-) to Sp7 (agg+pig-) (Lerner et al., 2010). Variant $Sp7_E$ displayed a different plasmid profile from its parental strain, but whether these modifications in plasmid composition are linked to phenotypic alterations of $Sp7_E$ remains to be established. In addition, the genetic elements involved in DNA rearrangements are still to be characterized. In the EPS-overproducing variants of Sp7 obtained on fructose medium, different genetic alterations using ERIC- and BOX-PCR as well as RAPD analysis could also be evidenced (Volfson et al., 2013).

As for variants of *A. lipoferum*, a *recA* mutant of strain *A. lipoferum* 4B was first shown to keep the ability to generate variants *in vitro*. The variants from 4B*recA* exhibited all morphological and biochemical features characteristic of the $4V_I$ variant (Vial et al., 2004). However, the frequency of variants generated by 4B*recA* appeared to be increased by up to 10-fold, contrasting with many studies demonstrating the abolition or a large reduction of the frequency of Pv in *recA* mutants. To assess whether genomic rearrangements take place during Pv of *A. lipoferum* 4B, RAPD profiles were obtained for the parental strain and the $4V_I$ variant: differential bands were obtained, providing the first evidence for DNA rearrangements (Vial et al., 2006). Using probes targeting the differential RAPD bands, it was shown that the genome of the $4V_I$ variant had undergone a deletion event. PFGE analysis with adapted conditions of migration and hybridization experiments revealed that a 750-kb replicon was missing in the $4V_I$ genome. The same rearrangements took place during Pv of 4B*recA*. Large-scale genomic rearrangements during Pv were demonstrated for two additional strains. In *A. brasilense* WN1, generation of stable variants was correlated with the disappearance of a replicon of 260 kb. For *A. irakense* KBC1, emergence of the unstable variant coincided with the formation of a new replicon of 160 kb, whereas the revertant recovered the parental genomic architecture (Vial et al., 2006).

The genome sequence of *A. lipoferum* 4B later revealed that the 750-kb replicon lost in the $4V_I$ variant was a chromid (AZOLI_p2), representing 11% of the genome and harboring 640 genes (Wisniewski-Dyé et al., 2011). Several genes or operons, lost during the process of Pv, can account for the phenotypic modifications observed in the $4V_I$ variant or the strain 4T such as *acdS* encoding ACC deaminase activity and the *nosRDZFYL* operon which catalyzes the reduction of nitrous oxide to dinitrogen (i.e., the last step of denitrification). A gene cluster consisting of five genes (*napABCDE*) which encode a periplasmic nitrate reductase is also present on the 750-kb replicon suggesting that the $4V_I$ variant is also affected in nitrate reduction to nitrite, a feature observed for strain 4T. Interestingly, several genes located within the 750-kb replicon are predicted to be involved in flagellin biosynthesis and are possibly the genetic determinants underlying the difference of motility observed between 4B and $4V_I$ variant. Finally, gene content analysis of the 750-kb replicon highlights numerous clusters of genes likely involved in siderophore production and iron and siderophore transport (e.g., a 14-kb region, AZOLI_p20158 to AZOLI_p20165 is predicted to be involved in pyochelin synthesis) (Wisniewski-Dyé et al., 2012). This observation may explain the slow growth rate of *A. lipoferum* 4T (and probably that of $4V_I$ variant) under low iron concentrations (Alexandre, 1998).

Several other studies have shown that plasmids of *A. brasilense* were involved in frequent and major genomics rearrangements; the resulting strains, although sometimes called variants, might simply be the result of genetic drift or mutations induced by stress. Interestingly, differences in genomic architecture of *A. brasilense* Sp245 strains obtained from different laboratories collections could be attributed to plasmid plasticity (number and size, integration in other replicons) (Pothier et al., 2008; see also Chapter 25). For *A. brasilense* Sp245, spontaneous mutants characterized by the loss of 2 plasmids of 85 and 120 MDa and the generation of a new replicon of more than 300 MDa have also been observed (Katsy et al., 2002). *A. brasilense* Sp7 also generated *in vitro* spontaneous mutants with altered plasmid composition, notably a modification of the size of the pRhico plasmid (Petrova et al., 2005).

103.5 OCCURRENCE OF PHENOTYPIC VARIATION IN NATURAL ENVIRONMENTS AND POTENTIAL IMPACT ON RHIZOSPHERE COMPETENCE AND PLANT GROWTH-PROMOTING PROPERTIES OF *Azospirillum*

It has been discussed almost since the rediscovery of *A. brasilense* in the 1970s by Tarrand et al. (1978) that some of the strains deposited in culture collections are very closely related, like the type strain *A. brasilense* $Sp7^T$, a strain initially isolated from rhizosphere soil of *Digitaria decumbens* in Brazil (Tarrand et al., 1978), with the strain *A. brasilense* Cd. The strain Cd was originally isolated from roots of plants [*Cynodon dactylon*] which had been inoculated with *A. brasilense* Sp7. Strain Cd has a characteristic red pigmentation, while colonies of strain Sp7 are only slightly pink. In the meantime, isolates with different carotenoid pigment content could be quite frequently derived from *A. brasilense* Sp7. The high carotenoid content resulted in increased oxygen tolerance of the variants (Hartmann and Hurek, 1988). It was demonstrated that a point mutation in the antisigma factor (Thirunavukkarasu et al., 2008) is responsible for this phenotypic alterations, which has implications for the oxygen tolerance of the bacterium (Hartmann and Hurek, 1988). Thus, the strain Cd could be a phenotypic variant of Sp7 generated in the rhizosphere.

During the course of isolating variants from strain *A. brasilense* Sp7- (agg-pig-), a variant has been isolated after colonization of maize roots in a gnotobiotic system; this variant displayed the phenotype agg-pig+EPS^{op}, with a significant higher EPS concentrations than the parental strain and a modified pattern for LPS. This indicates that Pv of *A. brasilense* may occur after inoculation and one might anticipate that overproduction of EPS could contribute to improved colonization of plant roots. When EPS-overproducing phenotypic variants of Sp7 were inoculated to maize, wheat, soybean, and peanuts in pot experiments with sterilized and nitrogen-free sand in the greenhouse, the EPS-overproducing variants showed in most cases the same extent of plant growth promotion effects as compared to the Sp7 wild-type strain (Volfson et al., 2013).

A. lipoferum 4B has been isolated simultaneously from the same rice rhizosphere and at the same frequency than *A. lipoferum* 4T, a nonmotile strain displaying laccase activity (Bally et al., 1983), and both strains displayed identical 16S rDNA sequences (Haurat et al., 1994). The plasmid profile of *A. lipoferum* 4T was compared to that of *A. lipoferum* 4B and revealed only two discrepancies: absence of the 750-kb replicon [like in the $4V_I$ variant] and presence of an additional replicon of 400 kb unrelated to the 750-kb replicon (Vial et al., 2006). Thus, *A. lipoferum* 4T could in fact be a variant of strain 4B generated within the soil ecosystem. Interestingly, after inoculation of rice roots with *A. lipoferum* 4B, nonmotile forms appeared in high numbers. However, no analysis of other specific phenotypes has been undertaken to identify these nonmotile forms (Alexandre et al., 1996).

Loss of swimming ability in the $4V_I$ variant and in strain 4T was directly linked to enhanced swarming motility (Alexandre et al., 1999b). Thus, the nonswimming *Azospirillum* strains are expected to keep the ability to move along plant roots. Whereas swimming motility is thought to play a role in chemotaxis of bacteria to plant root exudates, swarming across the surfaces of the roots may be important for long-term colonization. Interestingly, *A. lipoferum* 4T was shown to retain the ability to efficiently colonize rice roots (such a data is not available for the $4V_I$ and $4V_{II}$ variants) (Alexandre et al., 1996).

Bacterial laccases are considered as an advantageous trait for a rhizosphere bacterium as they are involved in various functions such as copper resistance, manganese oxidation, pigmentation, oxidation of toxic compounds, and destruction of reactive oxygen species (Sharma et al., 2007). Interestingly, the first report of a prokaryotic laccase is from the nonmotile isolate *A. lipoferum* 4T (Givaudan et al., 1993), where it was shown to play a role in melanization and utilization of plant phenolic compounds (Faure et al., 1994; 1996). Moreover, laccase-positive strains are less sensitive to the inhibitory action of quinone analogs due to rearrangements of their respiratory chain, a feature that might be a competitive advantage in the rhizosphere (Alexandre et al., 1999a).

The 750-kb chromid lost by the $4V_I$ variant carries genes that are relevant for interaction with plants, notably *acdS* that has been acquired by horizontal gene transfer (Prigent-Combaret et al., 2008; Wisniewski-Dyé et al., 2011). AcdS, by deaminating ACC, the immediate precursor of plant ethylene, is a key activity involved in the modulation of the plant hormonal balance by rhizobacteria. Because ethylene inhibits root growth and may be produced in too large amounts during plant stress response, bacterial ACC deamination can enhance both root system development and plant stress tolerance (Glick et al., 2007). *acdS* elimination might be useful to fine-tune the effect of *Azospirillum* on the plant hormonal balance; however, this hypothesis remains to be investigated. The plant hormonal balance might also be modulated by *nahG*, a gene located onto the 750-kb chromid (AZOLI_p20435) and contributing to the degradation of salicylate into catechol (Wisniewski-Dyé et al., 2012). As for nitrogen fixation, the relevant genes are all located onto the chromosome of strain 4B (see Chapter 25), suggesting the same nitrogen fixation capacity for 4B and $4V_I$ variant.

The question whether Pv modifies soil survival, rhizosphere competence, and plant growth-promoting properties of *Azospirillum* spp. deserves intense attention. Most recently, the EPS-overproducing variants of strain Sp7 were

shown to display higher stress resistance, and its colonization abilities to plant roots and plant growth-promoting effects were not influenced as compared to the wild type (Volfson et al., 2013). Nevertheless, from an applicative point of view, one can wonder if Pv of *Azospirillum* could be associated with some inconsistencies in plant growth promotion observed in the field. Also whether Pv could be manipulated to improve the fitness and thus the quality of the inoculants of this bacterium remains an open question. However, the occurrence of Pv has to be shown convincingly in field situation at many different sites and seasons.

103.6 PHENOTYPIC VARIATION IN NON-DIAZOTROPHIC ROOT-ASSOCIATED BACTERIA

The occurrence of phenotypic and phase variation in rhizosphere colonizing *Pseudomonas* bacteria has been reviewed previously (van den Broek et al., 2005). *Pseudomonas* sp. bacteria show Pv quite frequently, based on spontaneous mutation of the *gacA* and *gacS* genes. It was clearly demonstrated that site-specific recombinases are implicated in the initial genetic event of creating mutations in the *gacA/gacS* genes. In the case of the biocontrol bacterium *Ps. fluorescens* F113, a mutation in site-specific recombinases encoded by the *sss* and *xerD* genes generated phenotypic variants at a very low frequency compared to the wild type strain, both under laboratory conditions and after plant inoculation (Sánchez-Contreras et al., 2002; Martínez-Granero et al., 2005). In addition, these phenotypic variants were severely impaired in competitive root colonization. Most interestingly, it could be demonstrated that the expression of these recombinases was induced in the presence of the plant (Martínez-Granero et al., 2005).

Pv in *Pseudomonas brassicacearum,* a major root-colonizing population in *Arabidopsis thaliana*, has a major role in the root-colonizing strategy. During the colonization of *A. thaliana* and *Brassica napus*, bacteria can be retrieved with different colony appearance (Achouak et al. 2004). While wild-type cells (phase I cells) were located at basal parts of the roots, bacteria from translucent colonies (phase II cells) were essentially localized at young roots and root tips, a result obtained using differentially fluorescence-labeled phase I and phase II cells. Accordingly, phase II cells showed a higher ability to swim and to swarm, due to an overproduction of flagellin (Achouak et al., 2004). Comparison of the transcriptomes of these phase I and II cells revealed several genes relevant for secondary metabolism and small RNAs. In addition, it turned out that point mutations in the *gacA* and *gacS* genes accounted for the observed phenotypic switching to phase II cells. This was accompanied by downregulation of antifungal secondary metabolites, indole acetate, and exoenzyme (lipase and protease) production. Interestingly, also the production of three *N*-acyl-homoserine lactone (AHL; see Chapter 37) molecules was drastically reduced (Lalaouna et al., 2012). Since these are key regulators of the quorum-sensing response, some of the observed differences in phase II cells, like diminished biofilm formation, alginate biosynthesis, or expression of the type VI secretion machinery, could be a consequence of the altered AHL levels. Thus, in many *Pseudomonas*, the GacA/GacS regulatory system is of key importance for phenotypic/phase variation. While screening the published genomes of *Azospirillum*, no indication of a *gacA/gacS* similar genetic system has been found (Wisniewski-Dyé et al., 2011). Therefore, a similar mechanism as basis of phenotypic switching is not expected in *Azospirillum*.

The β-proteobacterium *Acidovorax radicis* N35, which was isolated from surface-sterilized roots of wheat, was shown to have growth-promoting effects on barley (Li et al., 2011). When plated on NB agar, phenotypic variants occurred with a high frequency of 3.2×10^{-3} per cell per generation (Li et al., 2012). While the wild type had a rough colony type, variant colonies (N35v) were characterized by their smooth appearance. Most strikingly, the variants showed almost no cell aggregation and had lost their flagella and swarming ability; moreover, the variants were no longer effective as plant growth-promoting bacteria in barley pot experiments in the greenhouse (Li et al., 2012). Inoculation of roots with a mix of differently fluorescence-labeled wild-type N35 and variant N35v cells demonstrated that the variant had lost its competitive root colonization abilities. When the genome sequences of the N35 wild type and the N35v variant were compared, one deletion could be found in all tested variants. The *mutL* gene which plays a key role in mismatch repair was affected by a 16-nucleotide deletion resulting in the expression of a truncated, nonfunctional MutL protein. The shift from root to laboratory conditions may have triggered this mutation by a hitherto unknown mechanism. This led to increased mutation frequency which resulted in an accumulation of point mutations, as could be shown in the comparison of the genome sequences of the wild type and the variant strain. Thus, this is an example that during laboratory cultivation of root-associated or rhizosphere bacteria, the potential of plant growth promotion can be lost. Therefore, Pv could be considered as one reason for less efficient PGPR performance after cultivation under laboratory conditions or during biotechnological cell mass production to obtain large inoculum biomass. Pv also occurs in plant pathogenic bacteria. Interestingly, in a recent study, Pv was observed in the *A. radicis*-closely related species *A. citrulli*, a serious pathogen of cucurbit plants (Burdman and Walcott, 2012). Pv in this pathogen was shown to be associated with loss of the ability to produce type IV pili [T4P] and with a significant reduction in virulence (Kumar Shrestha et al., 2013).

103.7 CONCLUSIONS

The occurrence of Pv has to be considered, when bacteria are isolated from their natural habitat, like the root environment. While some bacterial isolates may keep their original properties well under laboratory conditions, others undergo quite rapidly major changes, because genetic mutations and rearrangements may occur at high frequency. This genome plasticity may be considered as intrinsic genetic mechanism to improve plant growth promotion as well as challenge to preserve optimized rhizosphere competitivity (Terzaghi and O'Hara, 1990). In laboratory and biotechnological mass production, this dynamics of the genome should be taken into account and kept under control as much as possible. However, the genome plasticity could also be considered as a chance to preselect for strains optimized for specific niches. The "training" of candidate strains for biotechnological applications in the "rhizosphere school" could also be seen as a case of Pv and adaptive mutations towards optimized properties. Future studies in the fields will show whether this adaptation and optimization approach taking advantage of Pv is a possible way for strain improvement to specific plant and soil requirements. Furthermore, extensive comparative genome sequencing experiments of wild type and phenotypic variants will bring much more insights into the underlying molecular and genetic mechanisms in different bacterial species.

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Chapter 104

The Physiological Mechanisms of Desiccation Tolerance in Rhizobia

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104.1 INTRODUCTION

Poor survival of rhizobial inoculants on legume seed has long been recognized and is a result of several factors including desiccation (Deaker et al., 2004; see Chapters 39, 96). Significant losses in viable cell numbers occur during the drying and storage of coated seed with cell survival dependent on the rate of drying, water activity, and relative humidity during storage (Deaker et al., 2012; Hartley et al., 2012). Peat cultures of rhizobia applied to the surface of seeds or beads have a greater rate of survival than cells from agar or liquid cultures (Deaker et al., 2004; Bullard et al., 2005; Albareda et al., 2008). Dart et al. (1969) suggested improved survival was due to cells being partially encapsulated within a protective matrix of peat particles when coated onto seeds. However, cells extracted from peat cultures also survive better than liquid-cultured cells when coated onto plastic beads (Feng et al., 2002). Adaptive changes observed in peat-cultured cells such as cell wall thickening and changes in protein expression, believed to result from nutrient- and oxygen-limiting conditions during growth, have been suggested to contribute to their higher rate of survival (Dart et al., 1969; Feng et al., 2002).

Rhizobia accumulate intracellular compounds referred to as compatible solutes, or osmoprotectants, as a response to both osmotic and desiccation stress (Vriezen et al., 2007; see Chapter 96). Compatible solutes can be either synthesized *de novo* by rhizobia or transported into the cell from the environment via active uptake systems. The intracellular accumulation of trehalose in rhizobia has been observed to significantly improve desiccation tolerance (Streeter, 2003; McIntyre et al., 2007; Streeter, 2007). Trehalose is a nonreducing disaccharide and is believed to maintain membrane structure through the formation of biological glasses which protect proteins and other macromolecules from denaturation during desiccation stress (Crowe et al., 1984).

The biosynthesis of trehalose in *Rhizobium leguminosarum* bv. *trifolii* NZP561 involves the TreYZ and OtsAB pathways, and mutants deficient in either pathway accumulate trehalose in amounts similar to the wild type. However, double mutants fail to accumulate any trehalose and are also more sensitive to desiccation stress (McIntyre et al., 2007). The amount of accumulated intracellular trehalose varies between strains, species, growth stages, and conditions. Streeter (1985) reported that *Rhizobium japonicum* USDA110 and USDA138 accumulated more trehalose than USDA123 when ammonium nitrate was used as a source of nitrogen, compared with urea or glutamate. However, no explanation was given for this variation in trehalose accumulation as a result of the different nitrogen sources in the growth medium. In *R. leguminosarum* bv. *trifolii* NZP561, peak trehalose concentrations are reached in the early stationary phase of growth and decline during stationary phase due to catabolism as carbon in the growth medium becomes depleted (McIntyre et al., 2007).

Previous studies have reported that increasing the osmotic pressure of the growth medium through the addition of solutes such as sodium chloride increases the amount of accumulated trehalose in *R. leguminosarum* bv. *trifolii* TA1 (135 $\mu g\, mg^{-1}$ protein) and *Rhizobium meliloti* SU47 (280 $\mu g\, mg^{-1}$ protein) (Breedveld et al., 1991; Breedveld et al., 1993). Similarly, Streeter (2007) found that adding 40 mM sodium chloride to the medium, increased trehalose accumulation in bradyrhizobia by two- to threefold and

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that trehalose content gradually increased with further additions of sodium chloride up to 60 mM. However, growth of bradyrhizobia was significantly reduced when grown in basal medium containing 60 mM sodium chloride.

The addition of 3 mM trehalose to the growth medium increased intracellular trehalose concentration by threefold in cultures of *B. japonicum* USDA110. Increasing the exogenous trehalose concentration to 6 or 9 mM increased the trehalose content by five- to sixfold, respectively (Streeter, 2003). It was concluded that although USDA110 does not grow when trehalose is the sole source of carbon, it is able to accumulate the sugar at a slow rate in response to increased external osmotic pressure as a way of maintaining an isosmotic equilibrium. In a subsequent study, Streeter (2007) found that sodium chloride and trehalose had a cumulative effect when added to the medium. The addition of 40 mM sodium chloride and 3 mM trehalose resulted in a fivefold increase in trehalose accumulation in *B. japonicum*.

Evidence suggests that increased amounts of intracellular trehalose, whether synthesized or taken up by the cells from the growth medium, play an important role in desiccation tolerance. Survival of bradyrhizobia was highly correlated to the accumulation of increasing amounts of trehalose (Streeter, 2007). A two- to threefold increase in intracellular trehalose resulted in a 294% increase in survival of USDA110 after 24 h desiccation on soybean seed (Streeter, 2003).

In a separate study using NZP561, there was 70% recovery of cells immediately after air-drying when intracellular trehalose was synthesized by the wildtype. Intracellular trehalose was not detected in the *ots*A and *tre*Y double mutant, and cell recovery was 63%. Twenty-four hours after drying, recovery of viable cells was 39% for the NZP561 wild type and <0.001% for the double mutant, clearly indicating the role of trehalose in improved desiccation tolerance (McIntyre et al., 2007).

This study examined the growth conditions that affect trehalose accumulation in *R. leguminosarum* bv. *trifolii* (TA1) and *B. japonicum* (CB1809) and its role in desiccation tolerance. Physical and biochemical changes in cells exposed to the different growth conditions were then examined using vital staining, electron microscopy, and proteomic techniques.

104.2 METHODS

The osmotic pressure of JMM-defined medium (O'Hara et al., 1989) was altered through the addition of increasing concentrations of sodium chloride and trehalose. Osmotic pressure of the growth medium was estimated using Equation (104.1):

$$\pi = MRT \tag{104.1}$$

where π = osmotic pressure (atm), M = molarity of solutes (mol/L), R = ideal gas constant (0.0821 L atm/K mol), and T = Kelvin temperature (°C + 273) (Zumdahl, 1989).

Aqueous peat extract (50%, w/v) was used as a growth medium and was prepared by suspending gamma-sterilized peat in Milli-Q water. Cells of TA1 and CB1809 were grown aerobically in the different liquid media to early stationary phase and harvested through centrifugation. Desiccation tolerance was assessed by resuspending the harvested cells in water and drying under vacuum (Deaker et al., 2007). Trehalose accumulation at early stationary phase of growth was measured using a trehalose assay kit from Megazyme® according to the manufacturer's instructions. The effect of different growth media on membrane integrity was assessed using a LIVE/DEAD® BacLight™ Bacterial Viability Kit (L7012) according to the manufacturer's instructions. Cell morphology was examined by electron transmission microscopy using a JEOL 1400 electron microscope. SDS-PAGE was performed as described by Laemmli (1970), and peptide analysis carried out by liquid chromatography–mass spectrometry (LC–MS) using a C18 reversed-phase liquid chromatography coupled to a Q-TOF mass spectrometer. Details of the materials and procedures are as previously described by Casteriano et al. (2013).

104.3 RESULTS

104.3.1 Trehalose Accumulation in Rhizobia

Increasing the osmotic pressure of a defined growth medium (JMM) showed that trehalose accumulation increases as osmotic pressure increases (Fig. 104.1). Trehalose accumulation increased by 6- to 12-fold for TA1 and 3- to 5-fold for CB1809 in comparison to the control.

Trehalose accumulation was greater in TA1 and CB1809 cells grown in an aqueous peat extract compared to cells grown in the control medium (JMM). Cells of CB1809 grown in peat extract accumulated 150 ng of trehalose/μg of protein, which was 8-fold greater than that of cells grown in the control medium (JMM). Growing TA1 in crude peat extract increased trehalose accumulation by 5-fold in comparison to the amount accumulated by cells grown in the control medium (3.2 ng/μg protein).

104.3.2 Desiccation Tolerance in Rhizobia

The percentage of viable rhizobia immediately after drying was higher for cells grown at 2.5 atm compared to those grown in the control medium, as shown in Figure 104.2. However, this increase in survival was not found to be significant for either strain after analysis of variance (ANOVA).

Survival of TA1 was significantly improved immediately after drying and after 7 and 21 days in storage

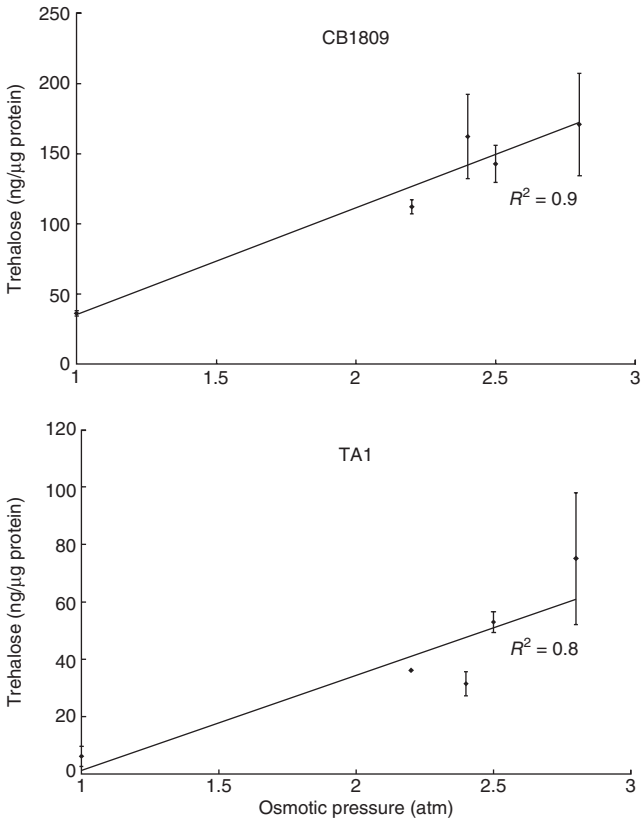

Figure 104.1 The effect of osmotic pressure on trehalose accumulation in rhizobia. Data points are the mean value of three replicate treatments and extractions ± SE (absence of error bars indicates that the error bar was less than the data point).

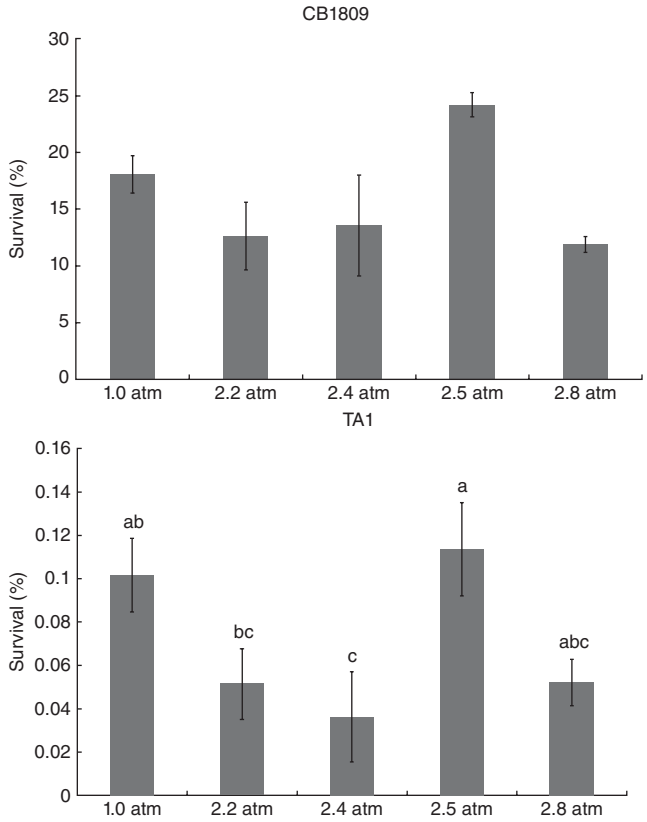

Figure 104.2 Survival of rhizobia immediately after vacuum drying. Cells were grown at increasing osmotic pressure with cells grown at 1.0 atm used as the control. Data shown are mean values of three replicates ± SE. Values with the same letter are not significantly different. Survival of CB1809 was not found to be significantly different ($p > 0.05$).

in 9% relative humidity when cells were grown in peat extract compared to that of the control. Survival of CB1809 grown in peat extract was not significantly improved after drying or during storage when compared to the control (Table 104.1).

104.3.3 The Effect of Growth Media on Membrane Integrity

A comparative analysis of the mean green-to-red fluorescence ratio (G/R_{Ratio}) between the growth media treatments was made using ANOVA for 10^5 cells of TA1 and 10^7 cells of CB1809 (Fig. 104.3). Analysis of the data showed that the ratio of TA1 cells grown in JMM medium (1.0 atm) was significantly higher than that of cells grown in the treatment media. No significant difference in the G/R_{Ratio} was found between cells grown at 2.5 atm and peat extract.

ANOVA of the G/R_{Ratio} for CB1809 showed that the ratio of cells grown at 2.5 atm was significantly higher than that of cells grown in the control and peat extract. The G/R_{Ratio} for cells grown in peat extract was significantly lower than that of cells grown in the control medium.

Table 104.1 The effect of growth medium on survival of Rhizobia after drying and storage

Strain and Growth Media	Initial	After Drying	7 Days in Storage	21 Days in Storage
TA1 (Control)	8.68	5.28	2.77	2.57
TA1 (Peat extract)	8.52	6.38	4.48	4.18
$P < 0.001$; LSD = 0.51				
CB1809 (Control)	9.08	8.36	7.50	7.32
CB1809 (Peat extract)	8.37	7.98	7.38	7.10
$P < 0.05$; LSD = 0.34				

The data are predicted mean values ($\log_{10}$ CFU/mL) of viable cells obtained using Residual Maximum Likelihood (REML) analysis

104.3.4 Changes in Cell Morphology and Protein Expression

Cells of TA1 grown in peat extract were found to be significantly smaller in length (0.87 µm) than cells grown in

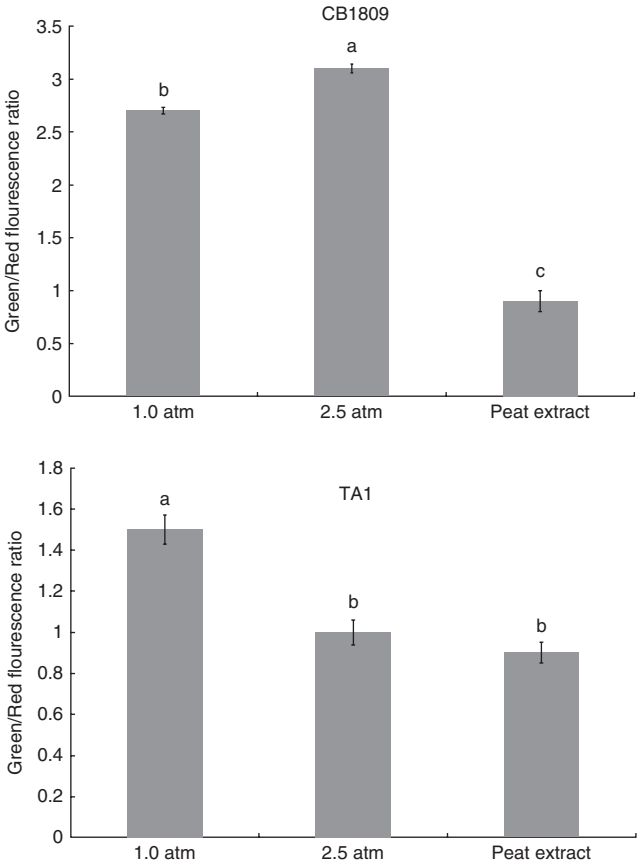

Figure 104.3 Membrane integrity assay using LIVE/DEAD staining. Values are the mean ratio of green-to-red fluorescence of three replicates ± SE. Columns with the same letter are not significantly different.

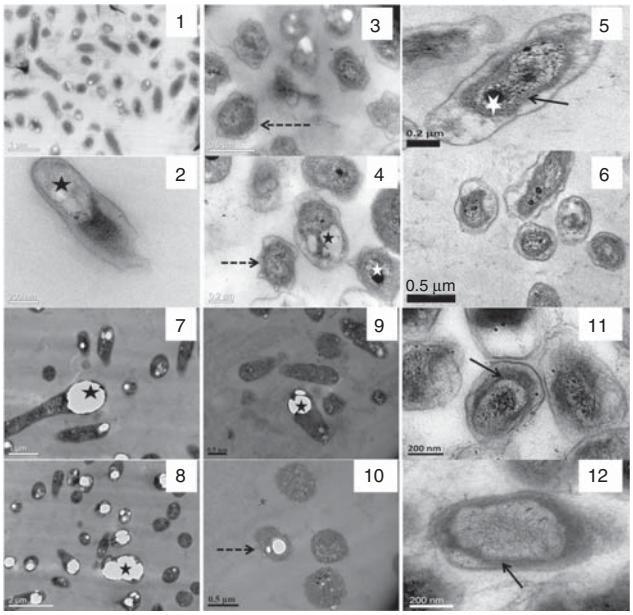

Figure 104.4 Transmission electron micrographs of rhizobia at early stationary phase of growth. 1 and 2 = CB1809 grown in JMM (1.0 atm); 3 and 4 = CB1809 grown in JMM (2.5 atm); 5 and 6 = CB1809 grown in crude peat extract; 7 and 8 = TA1 grown in JMM (1.0atm); 9 and 10 = TA1 grown in JMM (2.5 atm); 11 and 12 = TA1 grown in crude peat extract.

JMM media. The cell size of CB1809 grown in the treatment media was not significantly different to that of the control. The observed cells of rhizobia grown at 2.5 atm had noticeable undulations in the outer membrane, giving them the appearance of plasmolyzed cells, illustrated in Figure 104.4(3),(4),(9), and (10).

The cytoplasm of rhizobia grown in JMM media contained electron-dense inclusion bodies as well as abundant PHB granules (Fig. 104.4(2),(4),(7), and (9)). Although the cytoplasm of cells grown in peat extract was also found to contain electron-dense inclusions, PHB granules were less abundant than in cells grown in JMM media.

A concentration of electron-dense material was observed around the plasma membrane of cells grown in peat extract, giving the plasma membrane a more defined appearance (Fig. 104.4(5)(6)(11), and (12)).

Total protein extracts from cells of TA1 and CB1809 grown to early stationary phase in JMM media (1.0 and 2.5 atm) and peat extract were separated using one-dimensional (1D) SDS-PAGE (Fig. 104.5). Proteomic analysis showed that 25% of the bands analyzed for TA1 were downregulated in cells grown in the treatment media, while 67% were upregulated when compared to the control. Analysis of eight protein bands, differentially expressed in CB1809, showed that 63% of the bands were downregulated and 50% upregulated in the treatment media when compared to the control.

The comigration of proteins was identified in this analysis; therefore, it is difficult to conclude with certainty which protein or proteins are contributing to the observed changes in polypeptide band expression when rhizobia were exposed to stress. However, it is interesting to note the proteins that change in response to growth in the treatment media and to suggest possible mechanisms for their contribution to the observed improvements in desiccation tolerance. Among the peptides identified in the upregulated bands of rhizobia grown in the treatment media were proteins involved in the prevention and repair of oxidative damage, as well as proteins involved in the maintenance of membrane integrity (Table 104.2).

104.4 DISCUSSION

In this study, increased osmotic pressure of the growth medium increased trehalose accumulation in rhizobia which

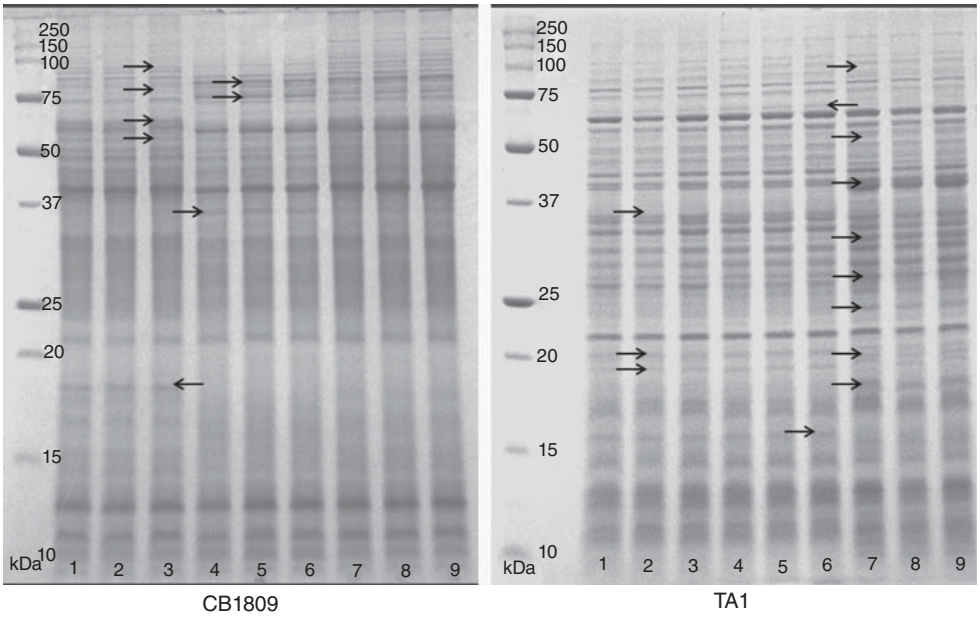

Figure 104.5 SDS-PAGE of total cell protein extracts from CB1809 and TA1 cells grown in different media. Cells of CB1809 were grown in JMM (lanes 4–6), JMM (2.5 atm) (lanes 7–9), and peat extract (lanes 1–3). Cells of TA1 were grown in JMM (lanes 1–3), JMM (2.5 atm) (lanes 4–6), and crude peat extract (lanes 7–9). Each lane corresponds to a single replicate growth treatment and protein extraction.

Table 104.2 Peptides of interest identified in response to growing rhizobia in treatment media (JMM 2.5 atm and crude peat extract) in comparison to the control

	CB1809		TA1	
	Protein	Function	Protein	Function
Upregulated	Lon protease	Protects DNA by degrading abnormal proteins	Phage shock (PspA)	Maintains membrane potential
	DNA-binding Dps family	Protects against oxidative stress	ATP-dependent Clp protease	Proteolytic activity
	60 kDa chaperonin	Prevents misfolding of peptides generated under stress	Thioredoxin	Oxidative stress protection
			Organic solvent Tolerance	Combats periplasmic stress
			60 kDa chaperonin	Prevents misfolding of peptides generated under stress
Downregulated	ABC transporter sugar binding	Transporter	Solute-binding component ABC	Transporter
	TonB receptor Hemin receptor	Iron transport	Ribosome recycling factor	Ribosome recycling after protein synthesis

supports previously published reports that rhizobia accumulate trehalose in response to osmotic stress (Breedveld et al., 1991; Breedveld et al., 1993; Streeter, 2003; Streeter, 2007).

The accumulation of intracellular trehalose by *B. japonicum* USDA110 was increased by supplying trehalose in the growth medium. In turn, the survival of trehalose-loaded cells was significantly greater than that of nonloaded cells 24 h after they were coated onto soybean seeds and air-dried (Streeter, 2003). McIntyre et al. (2007) reported that NZP561 (wild type) survived significantly better than the *ots*A and *tre*Y mutant strain after drying on glass beads and after storage for 24 hours at 5% and 32% relative humidity.

Our findings show that trehalose accumulation did not result in improved desiccation tolerance immediately after vacuum drying when cells were grown in defined medium with altered osmotic pressure. However, trehalose-loaded cells did survive significantly better after 10 days in storage at 9% relative humidity (data not shown).

Growing rhizobia in peat extract increased trehalose accumulation and survival of TA1 when compared to the control. Survival was not significantly improved for CB1809.

Although trehalose appears to play an important role in desiccation tolerance, the variation in survival of trehalose-loaded cells indicates that additional protective mechanisms are required to improve survival.

The results of the membrane integrity study showed that cells of TA1 and CB1809 grown in JMM medium have a greater ability to exclude propidium iodide (PI) than cells grown in peat extract. PI has been widely used in cell viability studies on the assumption that it is excluded from cells by intact membranes. According to this assumption, the results of this study infer that growing cells in peat extract compromises the integrity of membranes as the G/R_{Ratio} is significantly lower than that of the control cells. However, in this current study, growing rhizobia and in particular TA1 in peat extract was found to consistently increase desiccation tolerance.

Salema et al. (1982) reported that the process of drying and rehydration resulted in the rupture of the TA1 cell envelope causing death. Hence, to be able to withstand the process of drying and rehydration, cells must have a healthy cell envelope in order to remain viable. The reliability of PI as an indicator of membrane integrity must therefore be assessed keeping in mind that membrane integrity assays have been optimized for a limited number of bacterial species.

Previous reports on *Saccharomyces cerevisiae* indicate that cells under stress conditions can become more permeable to PI without loss of viability (Davey and Hexley, 2011). They found that exposing *S. cerevisiae* to heat stress resulted in 23% increase in PI uptake. However, removing the stress and allowing cells to recover for 1 hour prior to staining showed that only 14% of the cells remained permeable to PI. Similarly, cells exposed to ethanol and SDS became more permeable to PI, and removing the stressor prior to staining decreased PI uptake. They concluded that environmental stresses can cause reversible changes to membrane function and permeability, demonstrated by the changes in PI uptake.

Osmotic treatment of *S. cerevisiae* has been shown to lead to changes in membrane permeability resulting from the phase separation of phospholipids (Simonin et al., 2007). In this study, the cell envelope of TA1 was more permeable to PI after growth at higher osmotic pressure (2.5 atm). However, that was not the case for cells of CB1809 grown under the same osmotic conditions. It is difficult to suggest with certainty that cells grown in peat extract were also under osmotic stress as the osmotic pressure of that medium was unknown. Other chemical stressors present in peat may have been responsible for the possible changes to membrane permeability in those cells.

Heipieper et al. (1991) found that sublethal concentrations of phenolic compounds not only restricted growth of *E. coli* K12 but could increase membrane permeability. They observed the loss of ATP and nucleotides at both sublethal and lethal concentrations of phenolic compounds. Acidic phenols and tannins have been found to not only delay growth in *Lactobacillus plantarum* but also alter membrane fluidity. When acidic phenols were added to the medium, changes in the fatty acid composition of cells similar to that in response to low temperature and high alcohol concentrations were observed. Conversely, tannins added to the growth medium of *L. plantarum* had an effect on fatty acid composition and membrane fluidity comparable to that of increasing the temperature of the growth medium (Rozes and Peres, 1998). Henis et al. (1964) found that the tannin fraction of carob pod extract inhibited the growth of *C. fulvus*. Tannins are high-molecular-weight substances which form insoluble complexes with proteins. The mechanisms by which tannins inhibit growth were suggested to either be by adsorbing on their surface or by penetrating the cell wall and reacting with its components.

Peat is rich in phenolic compounds and tannins which would be expected to be also found in the extract. It is possible that these chemical constituents of peat affected the membrane permeability of rhizobial cells grown in the extract. PI uptake by rhizobial cells grown at 2.5 atm and in peat extract in this study may not indicate a decrease in membrane integrity as both growth treatments did not reduce their capacity for desiccation tolerance.

Transmission electron microscopy showed distinct changes in the cell morphology of rhizobia grown in peat extract. The accumulation of electron-dense material around the plasma membrane has previously been described in rhizobia extracted from peat (Dart et al., 1969; Feng et al., 2002). Those changes, believed to be an adaptive response to solid-state fermentation, have been suggested to contribute to their greater capacity for survival. Our findings suggest that the observed morphological changes are an adaptive response to the water-extractable constituents of peat.

Cytryn et al. (2007) reported on the genome-wide transcriptional analysis of *B. japonicum* USDA 110 in response to desiccation stress and concluded that trehalose accumulation, together with the expression of polypeptides such as membrane-protective proteins, DNA repair proteins, and oxidative stress responses, was necessary for desiccation tolerance.

Identification of proteins in rhizobia grown in crude peat extract and 2.5 atm showed an increase in proteins generated as a response to environmental stress compared with the control cells. Among several of the proteins identified in rhizobia grown in treatment media were two proteins

regulated by sigma factor o'^{E}, which typically regulates genes required to combat stress in the periplasmic space of *E. coli* (Abe et al., 2003; Aono et al., 1994; Dartigalongue et al., 2001). Phage shock protein A, or PspA, and OstA (organic solvent tolerance protein) are membrane-associated proteins. PspA maintains membrane potential by preventing proton leakage from damaged membranes (Kobayashi et al., 2007). It is expressed under membrane stress conditions such as growing cells in the presence of organic solvents, osmotic and heat stress (Kobayashi et al., 1998). OstA maintains cell envelope structure and is required for cell division, and its absence leads to loss of membrane integrity and consequently cell lysis (Braun and Silhavy, 2002). The increased expression of OstA and PspA may explain the morphological changes observed in the envelope of cells grown in crude peat extract and their increased capacity for desiccation tolerance. It could be suggested that physical damage to the cell envelope during drying and upon rehydration is minimized in peat extract-grown cells as a result of the maintenance of membrane potential and structure by OstA and PspA.

Other proteins induced in rhizobia after growth in crude peat extract and 2.5 atm include proteins which prevent DNA damage such as lon protease, thioredoxin, and DNA-binding stress response protein from the Dps family. Lon protease protects DNA from damage by degrading abnormal proteins, and ATP-dependent protease mutants of *E. coli* have been found to be more sensitive to DNA damage (Torres-Cabassa and Gottesman, 1987). Thioredoxin lowers reactive oxygen species levels (Hübner et al., 1996; Pauly et al., 2006), and DNA-binding stress response protein from the Dps family has been demonstrated to provide protection against oxidative stress (Chen and Helmann, 1995). Feng et al. (2002) also reported that superoxide dismutase was upregulated in peat cultures of rhizobia and suggested that this protein could be involved in improved survival through the protection of cells from oxidative damage.

Cytryn et al. (2007) found that genes induced in *B. japonicum* by desiccation stress encoded sugar transporter proteins and a 60 kDa chaperonin. Chaperonins have been shown to assist in the proper folding of misfolded or unfolded polypeptides generated under stress (Gutsche et al., 1999). These proteins were upregulated in both strains of rhizobia as a result of growth in crude peat extract in this current study, and their presence warrants further investigation in future proteomic or transcriptomic studies.

104.5 CONCLUSION

Desiccation tolerance in rhizobia may be enhanced through the manipulation of the growth medium. Trehalose accumulation, while playing an important role in desiccation tolerance, requires other poorly characterized processes to significantly improve survival. Growing rhizobia in peat extract appears to promote morphological changes and the upregulation of proteins involved in prevention and repair of desiccation-induced damage. This study provides a preliminary insight to the characterization of stress response protein expression which should be targeted through the use of more discriminatory techniques. Future work should also focus on the constituents of peat extract which appear to promote adaptive responses in rhizobia linked to enhanced desiccation tolerance.

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Chapter 105

Food Grain Legumes: Their Contribution to Soil Fertility, Food Security, and Human Nutrition/Health in Africa

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105.1 INTRODUCTION

In Africa, many grain legumes (both cultivated and wild) are consumed as food, especially during periods of food insecurity as a result of drought, flooding, or civil strife. The commonly cultivated food grain legumes include cowpea (*Vigna unguiculata* L. Walp.), Bambara groundnut (*Vigna subterranea* Verdc.), groundnut (*Arachis hypogea* L.), soybean (*Glycine max* L.Merr.), Kersting's bean (*Macrotyloma geocarpum* L.), and African yam bean (*Sphenostylis stenocarpa L.*). Many parts of these legumes are eaten as food. For example, the leaves and fresh mature pods of cowpea are eaten as a vegetable, while the dry grain is used in various food preparations. Nutritionally, there is about 35–40% protein in cowpea leaves; the grain also contains about 34% protein and 57% carbohydrate (Dakora, 2013).

Of the undomesticated grain legumes currently used as food security crops in Africa, fourteen are *Vigna* species. The tubers of *V. lobatifolia, V. reticulata*, *V. fischeri*, *V. vexillata, V. ambacensis, V. marina*, and *V. stenophylla* are harvested as food, in addition to the grain of *V. reticuleata*, *V. vexillata*, *V. juncea*, *V. gracilis*, *V. membranacea*, and *V, radiata,* which is consumed as pulse (Dakora, 2013). Nutritionally, the tubers of *V. lobatifolia, V. vexillata* and the African yam bean (*S. stenocarpa*) contain up to 15% protein, a level six times greater than that of cassava and three times than that of Irish potato or sweet potato (Dakora, 2013). Marama bean (*Tylosema esculentum* L.), an unimproved legume native to Southern Africa, produces grain containing 30–39% protein compared with the highly improved soybean with 38–40% protein and 43% oil compared with groundnut which has 48% oil (Dakora, 2013). Food legumes are high in dietary protein and fiber, but low in cholesterol, traits that are nutritionally ideal for good health.

In addition to their role as food in human nutrition, legumes are also used as high-protein feed and forage for livestock production. In many parts of the arid, semiarid and sahelian Africa, residues of food grain legumes are collected

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after harvest and used as feed for livestock during the dry season. Tree and shrub legumes are of particular importance as forage plants for animal production in arid, semi-arid and sahelian Africa. *Acacia albida, A. senegal*, and *A. karroo* are examples of leguminous species that serve as high-protein forage for livestock and wildlife, the latter being very important for ecotourism in Africa. Grain, shrub and tree legumes do serve as high-protein food and nutriceuticals for human consumption and high-protein fodder/forage for livestock production. This is because they display an ability to fix N_2 in root nodules when in association with specialized soil bacteria belonging to the genera *Rhizobium, Bradyrhizobium, Sinorhizobium* (*Ensife*r), *Azorhizobium*, and *Mesorhizobium*.

105.2 N_2 FIXATION AND N CONTRIBUTION BY FOOD GRAIN LEGUMES IN CROPPING SYSTEMS

In the past, measurements of N_2 fixation in nodulated legumes have largely centered on only a few genotypes or varieties, often leading to over generalization on their symbiotic performance under various agronomic/ecological conditions. As noted with quantification of N fixed, past inoculation studies often involving one or two varieties of grain legumes have also resulted in the generalized view that these species do not respond to inoculation. Today, many estimates of N_2 fixation by nodulated legumes in experimental field plots have tended to use a large number of legume genotypes (Table 105.1) with a greater chance of obtaining more representative results. Recent data on N_2 fixation and N contribution by food grain legumes in Africa, measured using the ^{15}N natural abundance technique, are summarized in Table 105.1.

The quantification of N_2 fixation by up to 32 cowpea varieties in Africa has revealed large differences in symbiotic functioning between and among genotypes. Although the 30 cowpea varieties planted at Taung in South Africa and at Wa and Manga in Ghana in 2005 were the same material, the percent N derived from fixation (%Ndfa) differed between locations and ranged from 8–60% at Manga in Ghana to 50–81% at Taung in South Africa and to 64–87% at Wa in Ghana (Table 105.1). The percent N obtained from fixation in 2005 for 15 genotypes planted at the three locations were 30–67% at Taung, 57–89% at Manga, and 56–96% at Wa. These %Ndfa values suggest that, in Africa, cowpea derives a large proportion of its N nutrition from symbiotic fixation.

Other grain legumes whose symbiotic performance has been assessed in Africa include groundnut, mungbean, pigeonpea, soybean, and chickpea. The evaluation of 25 groundnut varieties at three locations in 2010 revealed %Ndfa values ranging from 33–51% at Mzinti to 41–59% at Nelspruit and to 47–67% at Klipplaatdrift in South Africa. Fifteen field-grown mung bean genotypes also showed high levels of N_2 fixation at two locations, with %Ndfa values varying from 66–80% at Nelspruit to 71–86% at Klipplaatdrift in 2011 (Table 105.1), indicating once again that this species has a high dependency on N_2 fixation for its N nutrition. Similarly high levels of dependency (65–74%) on symbiotic N nutrition were obtained when 15 pigeon pea varieties were assessed for N_2 fixation at Nelspruit in South Africa (Table 105.1). Of the soybean varieties evaluated, the promiscuous-nodulating tropical glycine crosses derived between 54% and 63% of their N nutrition from symbiotic fixation in Ghana, while 13 strict-nodulating genotypes (which require rhizobial inoculation) obtained 39–83% of their N from fixation at Vredefort, 60–96% at Balfour, and 60–96% at Bergville in South Africa (Table 105.1; see also Chapter 99). While more studies are needed to properly compare the performance of the two soybean types in Africa, the promiscuous-nodulating genotypes appear to produce less symbiotic N relative to the strict-nodulating varieties. Twenty-six genotypes of chickpea (kabuli) also exhibited large differences in their dependency on N_2 fixation for N nutrition, with %Ndfa values ranging from 3–91% at Klipplaatdrift to 14–86% at Nelspruit and to 27–72% at Mzinti in the Mpumalanga Province (Table 105.1), indicating genotypic variation in symbiotic response to the different sites.

The variation in N_2 fixation observed between legume species and genotypes in the different experimental environments could relate to biotic and abiotic factors, which include the soil moisture and temperature, the level of mineral nutrients in plant rhizosphere, the soil mineral N, the varietal differences, as well as the presence, size, and efficacy of root nodule bacterial populations in the soil (Dakora and Keya, 1997). There is evidence to show that rhizobial biodiversity is greater where legume diversity is high (Pule-Meulenberg et al., 2010). This was exemplified in a recent study, where we found greater cowpea rhizobial diversity in South Africa compared to Ghana and Botswana, which have a relatively low plant biodiversity compared to the high legume diversity and endemicity in South Africa (Pule-Meulenberg et al., 2010). Furthermore, rhizobial IGS type symbiotic efficiency can differ considerably even within the same location. Estimates of rhizobial strain IGS type symbiotic efficiency revealed marked differences in symbiotic N yield independent of single or multiple nodule occupancies (Pule-Meulenberg et al., 2010). For example, strain IGS type XI produced greater symbiotic N in soybean genotype TGx1445-3E as sole nodule occupant, in contrast with nine strain IGS types in root nodules of the highly promiscuous genotype Salintuya-1 that produced less symbiotic N (Pule-Meulenberg et al., 2010).

As found for %Ndfa, there was a large variation in N contribution by the different genotypes of cowpea, soybean, pigeon pea, groundnut, and mung bean evaluated in field

Table 105.1 Recent data on N_2 fixation and N contribution by grain legumes sampled from experimental plots in Africa, measured using ^{15}N natural abundance

Legume Species	Year	No. of Genotypes	Location	%Ndfa	N Contribution (kg ha^{-1})	Grain Yield (kg ha^{-1})	References
Cowpea	2005	32	Taung (RSA)	50–81	48–182	200–3400	Belane et al. (2011)
	2005	30	Manga (Ghana)	7.9–60	8–149	221–1030	Belane and Dakora (2008)
	2005	30	Wa (Ghana)	64–87	56–178	50–1090	Belane and Dakora (2009)
	2006	15	Manga (Ghana)	57–89	17–169	734–1495	Belane and Dakora (2009)
	2006	15	Wa (Ghana)	56–96	62–198	1100–2400	Belane and Dakora (2009)
	2006	15	Taung (RSA)	30–67	24–131	1200–3500	Belane et al. (2011)
Soybean	2006	6	Wa (Ghana)	54–63	79–122	na	Pule-Meulenberg et al (2010)
	2008	7	Nampula (Mozambique)	44–73	19–31		Gyogluu and Dakora (unpubl. data)
	2009	7	Ruace (Mozambique)	79–90	153–240	na	Gyogluu and Dakora (unpubl. data)
	2009	7	Mutequelesse (Mozambique)	50–55	36–62	na	Gyogluu and Dakora (unpubl. data)
	2010	20	Potchefstroom (RSA)	50–78	86–350	na	Mapope and Dakora (unpubl. data)
	2011	13	Balfour (RSA)	60–96	82–220	1600–2700	Mapope and Dakora (unpubl. data)
	2011	13	Bergville (RSA)	50–97	23–282	1400–2800	Mapope and Dakora (unpubl. data)
	2011	13	Vredefort (RSA)	39–83	95–347	1800–3000	Mapope and Dakora (unpubl. data)
Pigeon pea	2010	15	Nelspruit (RSA)	65–74	36–144	na	Murwa and Dakora (unpubl. data)
	2011	12	Klipplaatdrift (RSA)	27–55	42–156	na	Phatlane and Dakora (unpubl. data)
	2011	12	Nelspruit (RSA)	82–92	106–151	na	Phatlane and Dakora (unpubl. data)
	2012	12	Mzinti (RSA)	79–91	88–400	na	Phatlane and Dakora (unpubl. data)
Groundnut	2010	25	Nelspruit (RSA)	41–59	76–188	469–1423	Mogkehle and Dakora (unpubl. data)
	2010	25	Klipplaatdrift (RSA)	47–67	70–171	304–1096	Mogkehle and Dakora (unpubl. data)
	2010	25	Mzinti (RSA)	33–51	58–126	944–2051	Mogkehle and Dakora (unpubl. data)
	2012	16	Gerbi (Ethiopia)	24–40	60–105	598–2069	Muhaba and Dakora (unpubl. data)
	2012	3	Bedeno (Ethiopia)	40–43	137–156	1300–1400	Muhaba and Dakora (unpubl. data)
	2012	21	Damango (Ghana)	32–50	46–134	na	Muhaba and Dakora (unpubl. data)
Mung bean	2011	15	Nelspruit (RSA)	66–80	53–84	300–700	Mokobane and Dakora (unpubl. data)
	2011	15	Klipplaatdrift (RSA)	71–86	31–111	100–400	Mokobane and Dakora (unpubl. data)
Chickpea (kabuli)	2011	26	Mzinti (RSA)	27–72	1–8	na	Makhura and Dakora (unpubl. data)
	2011	26	Klipplaatdrift (RSA)	3–91	0.1–5	na	Makhura and Dakora (unpubl. data)
	2011	26	Nelspruit (RSA)	14–86	2–16	na	Makhura and Dakora (unpubl. data)
	2012	26	Klipplaatdrift (RSA)	20–74	3–16	na	Makhura and Dakora (unpubl. data)
	2012	26	Nelspruit (RSA)	20–92	2–17	na	Makhura and Dakora (unpubl. data)
	2012	26	Sutherland (RSA)	39–82	4–34	na	Makhura and Dakora (unpubl. data)
Chickpea (desi)	2012	40	Mzinti (RSA)	27–75	1–7	na	Makhura and Dakora (unpubl. data)
	2012	40	Nelspruit (RSA)	24–72	3–20	na	Makhura and Dakora (unpubl. data)
	2012	40	Klipplaatdrift (RSA)	21–82	1–6	na	Makhura and Dakora (unpubl. data)

na = not available.

Table 105.2 Recent data on N_2 fixation and N contribution by grain legumes grown in farmers' fields in Africa, measured using ^{15}N natural abundance

Legume Species	Year	Study Site	%Ndfa	N Contribution (kg N ha^{-1})	References
Bambara groundnut	1999	Southern Zambia	49	21	Nyemba and Dakora (2010)
	1999	Central Zambia	72	34	Nyemba and Dakora (2010)
	1999	Northern Zambia	63	68	Nyemba and Dakora (2010)
	2005	Botswana	36–40	nd	Dakora et al. (unpubl. data)
	2009	South Africa	33–98	4–200	Dakora et al. (unpubl. data)
Groundnut	1999	Southern Zambia	27	19	Nyemba and Dakora (2010)
	2005	Botswana	32–47	nd	Pule-Meulenberg et al. (unpubl. data)
Cowpea	1999	Southern Zambia	60	64	Nyemba and Dakora (2010)
	1999	Central Zambia	59	35	Nyemba and Dakora (2010)
	1999	Central Zambia	31	43	Nyemba and Dakora (2010)
	1999	Northern Zambia	70	79	Nyemba and Dakora (2010)
	2005	Ghana (Nadowli district)	90	17	Naab et al. (2009)
	2005	Ghana (Sissala district)	79	23	Naab et al. (2009)
	2005	Ghana (Jirapa district)	83	18	Naab et al. (2009)
	2005	Ghana (Wa district)	66	19	Naab et al. (2009)
	2005	Ghana (Lawra district)	79	21	Naab et al. (2009)
	2005	Botswana	13–87	nd	Pule-Meulenberg and Dakora (unpubl. data)
	2006	Botswana	29–92	nd	Pule-Meulenberg and Dakora (unpubl. data)
	2006	Botswana	40–66	nd	Pule-Meulenberg and dakora (unpubl. data)
Common bean	1999	Central Zambia	55	6	Nyemba and Dakora (2010)
	1999	Northern Zambia	3	1	Nyemba and Dakora (2010)
Soybean	2010	South Africa	32–96	18–298	Mapope and Dakora (unpubl. data)

nd = not determined.

experiments (Table 105.1). Where 30 cowpea genotypes were assessed at three locations in Ghana and South Africa, in general, the data showed greater symbiotic N yield at Wa in Ghana in 2005 and 2006, followed by Taung in South Africa and then Manga in Ghana (Table 105.1). Some cowpea genotypes such as IT84S-2246, Glenda, Fahari, and IT90K-59 were found to be high grain yielding and high N_2 fixers across the three locations. The genotype IT84S-2246 could, for example, produce as much as 146, 175, and 111 kg N ha^{-1} at Wa, Manga, and Taung, respectively. The genotype Glenda also fixed 109 and 102 kg N ha^{-1} at Wa and Taung, Fahari 105 and 159 kg N ha^{-1} at Manga and Taung, and IT90K-59 106 and 114 kg N ha^{-1} at Wa and Manga, respectively. These high-yielding, high N_2-fixing cowpea genotypes could serve as food security crops as well as biofertilizers for improving soil fertility in African cropping systems. There was a similar pattern in N contribution by 13 soybean, 25 groundnut, 15 mung bean, and 26 chickpea (kabuli) genotypes evaluated at two to three locations in South Africa (Table 105.1). Unlike the other genotypes, soybean and chickpea were inoculated to ensure nodulation and N_2 fixation. Although they exhibited high levels of symbiotic dependency on N_2 fixation for their N nutrition (up to 72, 86, and 92% at the three different experimental sites), the 26 chickpea genotypes evaluated in South Africa recorded the lowest amounts of N fixed (0.1–34 kg N ha^{-1}) when compared to other legume species, indicating that this grain legume is less likely to make a significant contribution to the N economy of cropping systems in Southern African region.

Some studies have also been carried out on N_2 fixation and N contribution by grain legumes in farmers' fields in the African continent. The food legumes studied under farmer conditions include cowpea, Bambara groundnut, soybean, groundnut, and common bean (Table 105.2). On average, Bambara groundnut could derive between 49% and 72% of its N nutrition from symbiosis in farmers' fields in Zambia, 33% to 98% in South Africa, and 36% to 40% in Botswana, while cowpea obtained 31–70% in Zambia and 13–87% in 2005 and 29–92% in 2006 in Botswana (Table 105.2). The proportion of N derived from the atmosphere by soybean for its N nutrition was 32–96% in South Africa and groundnut 32–47%. Despite the relatively high levels of dependency on N_2 fixation for the N nutrition of grain legumes from farmers' fields, their N contribution was generally low (Table 105.2) due to the low legume plant density in the cropping system. With cowpea in Ghana, for example, plants derived as much as 66-90% of their N nutrition from symbiosis in farmers' fields and yet contributed only 17–23 kg N ha^{-1} (Table 105.2). This suggests that the low N contribution is not from poor symbiotic functioning, but

rather from low legume plant density, which affected dry matter yield.

105.3 N_2-FIXING EFFICIENCY AND MINERAL ACCMULATION IN GRAIN LEGUMES

In addition to N_2 fixation, symbiotic legumes are also reported to accumulate greater levels of mineral nutrients in their tissues than cereals (Pederson et al., 2002; Broadley et al., 2003; Fageria, 2004). More recently, Belane and Dakora, 2011a,b, 2012 have shown that cowpea can accumulate more macro-/micronutrients in its leaves compared to spinach, a commercial vegetable grown in South Africa. As a result of these reports (Pederson et al., 2002; Broadley et al., 2003; Fageria, 2004; Belane and Dakora, 2011a,b, 2012), a field study was recently conducted at Wa in Ghana to establish the relationship, if any, between N_2 fixation and mineral accumulation in nodulated cowpea. The data showed that the cowpea genotypes, which produced the largest amounts of symbiotic N, accumulated the most mineral nutrients in organs. For example, the genotypes with superior symbiotic performance consistently exhibited greater accumulation of P, K, Mg, S, Na, Fe, Cu, Zn, Mn, and B in young fully expanded trifoliate leaves (Table 105.3), clearly indicating a link between legume N_2 fixation and mineral accumulation in organs.

Many soil microbes are capable of solubilizing mineral nutrients in plant rhizosphere for increased uptake by roots (Vessey, 2003). There are, for example, reports of bacterial endophytes that promote uptake of mineral nutrients such as P, Fe, and Zn in the rhizosphere (Barretti et al. 2008). Other studies have also found an increase in N, P, K, Ca, Mg, B, Mn, Zn, Fe, Cu, Al, and Na in organs of inoculated groundnut (Howell, 1987). Furthermore, applying a strain of *Mesorhizobium mediterraneum* to the roots of chickpea increased tissue concentration of K, Ca, Mg, P, and N (Peix et al., 2001), a result similar to inoculating *Phaseolus vulgaris* plants with *Burkholderia cepacia* (Peix et al., 2001). An increase in the concentration of trace elements in *Phaseolus vulgaris* was also observed, following inoculation with rhizobial bacteria (Ndakidemi et al., 2011). From these reports, it appears that many nonrhizobial bacteria are capable of solubilizing mineral nutrients in the rhizosphere (Vessey, 2003) and promoting increased mineral uptake. Experiments conducted under strict microbiologically controlled environments are therefore needed to validate these reports of bacteria-induced mineral accumulation (Howell, 1987; Peix et al., 2001; Ndakidemi et al., 2011).

One such experiment was done by applying seven infective rhizobial strains to the seedlings of cowpea (cv. TVu11424) grown in sterile Leonard jars. The results showed marked differences in plant growth and symbiotic efficacy between strains (Table 105.4). Strains TUT53b2vu and TUT33b4vu were more efficient in N_2 fixation, measured as dry matter yield (Table 105.4). A 0.5 mM NO_3-fed treatment also produced significantly higher plant growth than the remaining five rhizobial isolates. ICP-MS analysis of nutrients in the nodulated cowpea plants revealed marked differences in mineral accumulation in shoots. Strains TUT26a1vu and TUT13d1vu, which were symbiotically more efficient (measured as dry matter yield), recorded significantly higher content of P, K, Ca, Mg, S, Fe, Cu, Mn, and B in cowpea shoots (Table 105.5). In contrast, strain TUT25d1vu, which showed the lowest symbiotic efficiency, also induced the least mineral accumulation in host plant shoots (Table 105.5). The 0.5 mM NO_3^--fed cowpea plants exhibited the next higher amounts of minerals after the

Table 105.3 Plant Growth, Amount of N-fixed, and Macronutrient Levels in Cowpea Leaves Sampled at 46 DAP at Wa, Ghana in 2005

Genotypes	Plant Biomass (kg ha^{-1})	N Fixed (kg N ha^{-1})	Concentration (mg/g)				
			P	K	Mg	S	Na
High N Fixers							
IT90K-76	3700b	123b	6.6a	36.5a	4.4a	1.7b	354ab
Bensogla	3967b	144a	5.5b	34.7b	4.8a	2.0a	308ab
Glenda	4618a	147a	5.5b	34.1b	4.4a	1.7b	378a
Low Fixer							
ITH98-46	1468c	49c	2.8c	27.0c	3.6b	1.5c	250c
			Amounts (mg/leaf)				
IT90K-76			0.15a	15.7a	14.1ab	0.88a	0.06ab
Bensogla			0.12b	14.7a	14.2ab	0.73b	0.07b
Glenda			0.12b	14.9a	12.7b	0.70b	0.06b
ITH98-46			0.06c	11.6b	10.6c	0.68c	0.04c

Values (Mean ± SE, $n = 30$) followed by dissimilar letters in a column are significantly different at $P < 0.05$.

Table 105.4 Nodulation and dry matter yield of cowpea seedlings inoculated with seven rhizobial strains

	Nodulation		Dry Matter		
Strain	Nod no. Per Plant	Nod Fwt g.plant^{-1}	Shoot g.plant^{-1}	Root	Whole Plant
TUT53b2vu	40 ± 3a	0.9 ± 0.1a	2.3 ± 0.3a	0.6 ± 0.1a	3.2 ± 0.40a
TUT33b4vu	38 ± 1a	0.8 ± 0.1b	1.9 ± 0.2a	0.2 ± 0.0b	2.5 ± 0.21a
TUT15a3vu	27 ± 1b	0.5 ± 0.0cd	0.3 ± 0.1b	0.2 ± 0.0b	0.6 ± 0.15b
TUT81a1vu	24 ± 6bc	0.4 ± 0.0cd	0.2 ± 0.0b	0.1 ± 0.0b	0.5 ± 0.04b
TUT26a1vu	22 ± 2bc	0.3 ± 0.0de	0.2 ± 0.0b	0.1 ± 0.0b	0.3 ± 0.03b
TUT13d1vu	18 ± 2c	0.2 ± 0.0e	0.3 ± 0.0b	0.2 ± 0.0b	0.5 ± 0.05b
TUT25d1vu	39 ± 1a	0.6 ± 0.0b	0.2 ± 0.0b	0.2 ± 0.1b	0.4 ± 0.11b
0.5 mM NO_3^-	na	na	1.9 ± 0.4a	0.5 ± 0.1a	2.4 ± 0.5a

Values (Mean ± SE) followed by dissimilar letters in a column are significantly different at $P < 0.05$. na = not applicable.

Table 105.5 Amounts of macronutrients and trace elements in shoots of cowpea plants inoculated with seven rhizobial strains

Strain	P mg/shoot	K	Ca	Mg	S	Fe µg/shoot	Cu	Zn	Mn	B
TUT53b2vu	13 ± 2a	107a	30a	14a	15a	605a	13a	134	226a	138a
TUT33b4vu	4 ± 0b	78b	22b	8b	7b	250b	11abc	75	139b	114ab
TUT15a3vu	1 ± 0c	16c	4d	2c	1c	48b	3bc	18bc	47c	19c
TUT81a1vu	0.7 ± 0c	8c	3d	1c	1c	25b	2c	10c	36c	14c
TUT26a1vu	0.7 ± 0c	11c	4d	1c	2c	28b	3bc	10bc	33c	15c
TUT13d1vu	1.1 ± 0c	16c	6d	2c	3c	88b	4bc	20bc	73c	31c
TUT25d1vu	0.5 ± 0c	9c	3d	1c	1c	30b	1c	10c	33c	14c
0.5mMNO_3^-	1.9 ± 0bc	87ab	14c	6b	3c	212b	21a	80a	96b	88b

Values (Mean ± SE, $n = 30$) followed by dissimilar letters in a column are significantly different at $P < 0.05$.

two best-performing strains TUT53b2vu and TUT33b4vu (Table 105.5).

105.4 CONTRIBUTION OF LEGUME SYMBIOSIS TO FOOD SECURITY AND ENHANCED HUMAN NUTRITION/HEALTH

The ability of nodulated legumes to reduce atmospheric N_2 into NH_3 in root nodules using the enzyme nitrogenase is crucial to global food security, human nutrition, and health (see Chapter 109). The excess NH_3 produced, following utilization by bacterial cells, is converted into amino acids via the GS/GOGAT pathway inside the host plant. The synthesis of amino acids and other nitrogenous solutes from symbiotic N forms the basis for the high protein levels in grain and leaves of nodulated legumes, and therefore, the higher the N_2 fixation in a legume, the greater the protein concentration/content in plant organs. But more importantly, the higher the N_2 fixation in grain legumes, the greater the plant growth, biomass accumulation, and grain yield (Belane and Dakora, 2009; Pule-Meulenberg and Dakora, 2009; Makoi et al., 2009; Belane and Dakora, 2010; Belane et al., 2011), thus indicating the contribution of N_2 fixation to legume grain yield and ultimately to food security.

However, as indicated earlier, in addition to N_2 fixation, nodulated legumes also accumulate mineral nutrients in organs, a trait much needed in African cropping systems. African agriculture is generally faced with soil infertility, leading to low crop yields, as well as food and nutritional insecurity, which in turn cause protein–calorie malnutrition with components of trace element deficiency, kwashiorkor in children, and diabetes in adults. In our view, symbiotic N_2 fixation in grain legumes is the key to overcoming the food security and human nutrition/health problems in Africa. This is because N_2 fixation can contribute to soil N economy in cropping systems, increase soil fertility (other than N), improve plant/animal protein production, raise yield levels of legume and cereal crops, as well as overcome a host of nutritional disorders, including trace element deficiency. The fact that symbiotic activity in nodulated legumes is linked to mineral accumulation in these species has implications for human nutrition and health, including micronutrient deficiency, which is a major problem in Africa. In addressing this nutritional disorder, the South African government has resorted to exogenous supplementation of maize flour ("mealie meal") with Fe, Zn, and Se in order to reduce trace

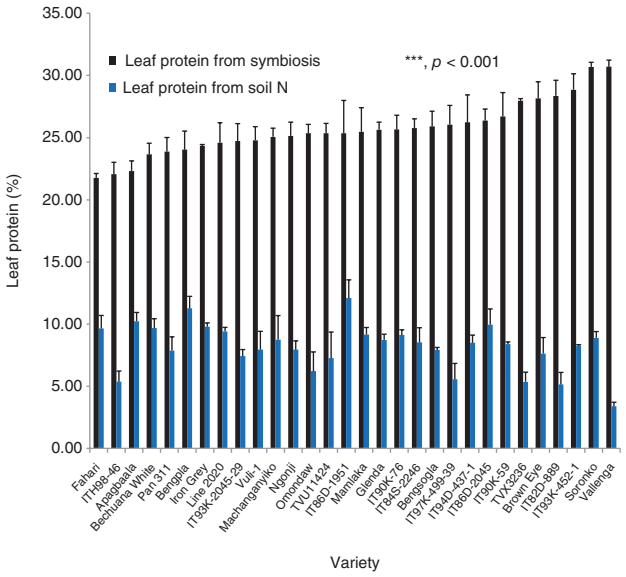

Figure 105.1 Comparison of cowpea protein from symbiosis and soil at Wa, Ghana, in 2003.

element deficiency in the population (Belane and Dakora 2011a). While conventional plant breeding and genomic approaches have been employed elsewhere to promote micronutrient uptake by crop plants, and thus increase mineral nutrients in grain in order to overcome trace element deficiency in consumers (Welch and Graham, 2004; Bouis, 2003), our data (Tables 105.3–105.5) suggest that the same can be achieved by identifying combinations of rhizobia and food grain legumes that fix greater amounts of symbiotic N (Tables 105.1–105.3) and accumulate mineral density in edible plant parts (Table 105.3) for improving human nutrition and health.

As indicated previously, in addition to enhanced mineral supply, the legume/rhizobia symbiosis is also a huge source of dietary protein for human consumption. Figure 105.1 shows a comparison of leaf protein originating from symbiosis and that from soil N in cowpea leaves. To estimate leaf protein from the two sources, symbiotic N and soil N were measured in cowpea leaves using the ^{15}N natural abundance technique and %N fixed (the dividend of N fixed and leaf dry matter) multiplied by 6.25 to obtain leaf protein from symbiotic origin. The leaf protein from soil source was obtained by multiplying %N from soil by 6.25. The data clearly show that where the legume was deriving a high proportion of its N nutrition from symbiosis (85–95% Ndfa and 5.1–6.3% N in cowpea leaves at Wa), most of the leaf protein synthesis was dependent on symbiotic N, with very little coming from soil N.

In this study, nodulated cowpea could produce as much as 22 to 32% of its leaf protein from N_2 fixation, indicating that, with cowpea, rhizobial symbiosis does contribute significantly to food and nutritional security, as well as to improved human nutrition and health. Additionally, by being high in protein and fiber, and low in cholesterol, grain legumes is a source of healthy diet for the rural poor in Africa.

In conclusion, with the selection of high N_2-fixing rhizobia and high-yielding hosts, food grain legumes in Africa have the potential to increase soil fertility and double crop productivity in the continent, eradicate hunger and food poverty, eliminate micronutrient deficiency and kwashiorkor in children, reduce protein–calorie malnutrition in rural communities, and enhance the overall human nutrition and health while creating cash and wealth for farmers. More studies are however needed to intensify the search for both high-yielding legume genotypes and high-performing rhizobial symbionts for the different agroecological zones of Africa, especially with adaptability to climate change. In our view, symbiotic N_2 fixation is the solution to Africa's food and nutritional security problems.

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Chapter 106

Plant Breeding for Biological Nitrogen Fixation: A Review

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106.1 INTRODUCTION

Costs associated with nitrogen fertilizers have increased steadily and are predicted to rise into the future (Tilman, 1999). There are also environmental concerns associated with gaseous emissions, and fertilizer inefficiency has resulted in an increased interest in biological nitrogen fixation (Peoples et al., 1995; see Chapter 108). Nitrogen-fixing crops (crop legumes) naturally replenish soil nitrogen without the use of nitrogenous fertilizers. This ability is facilitated by a complex symbiotic relationship between nitrogen-fixing bacteria, comprising 13 different genera known as rhizobia (Willems, 2006), and a host plant, culminating in the establishment of root nodules.

Plant-related nitrogen fixation is a significant contributor to the global agricultural nitrogen budget, adding 21.45 million tons of fixed N each year (Herridge et al., 2008; see Chapters 5 and 109). Increasing the effectiveness of N fixation in agricultural systems could be a powerful tool in reducing reliance on chemical fertilizers and sustainably increase agricultural production. Nevertheless, while there are various benefits of improved N fixation, attempts at targeted breeding have been minimal (Herridge and Rose, 2000). Genetic diversity for traits related to enhanced N fixation has been identified in soybean (Nicolas et al., 2002; Hungria and Bohrer, 2000), field pea (Buttery et al., 1992), model species *Medicago truncatula* (Rangin et al., 2008), common bean (Redden and Herridge, 1999; Rodino et al., 2005), chickpea (Biabani et al., 2011), and others.

The heritability of symbiotic traits and the resulting opportunity to realistically breed for N fixation have also been documented. Reviewing heritability of N fixation traits on a range of legume crops, Provorov and Tikhonovich (2003) concluded that in many cases the broad-sense heritability of N fixation may be as high as 0.90, narrow-sense heritability as high as 0.76, and realized heritability up to 0.96, all strongly suggesting that breeding for enhanced symbiosis is a realistic target. Nicolas et al. (2002), studying soybean (*Glycine max*) populations derived from four commercial cultivars, found the narrow-sense heritability of nodulation characteristics (nodule number and nodule weight) to range from 0.39 to 0.77, illustrating the potential gains in nodulation when considered parent selection is made. Also studying soybean, Gwata et al. (2004) suggested that the genes controlling indiscriminate or promiscuous nodulation may be relatively few in number. To demonstrate this, soybean cultivars were inoculated in a glasshouse experiment with cowpea (*Vigna unguiculata*) rhizobia strains, where nodulation indicated promiscuity. Promiscuous and nonpromiscuous lines were then crossed to assess heritability based upon nodule dry weight and leaf color score. Their findings suggested that nonpromiscuity was partially dominant [h/d = 0.37] for nodule dry weight and almost completely dominant for leaf color score [h/d = 0.74]. Working with peanut (*Arachis hypogaea*), Sikinarum et al. (2007) crossed high- and low-fixing cultivars and generated six F_5 populations to assess heritability of nodule weight, leaf color score and shoot dry weight. Generally, broad-sense heritability was high (>0.50) for most fixation-associated traits with nodule dry weight ranging from 0.63 to 1.0 and fixed nitrogen ranging from 0.85 to 0.98. However, Herridge and Danso (1995) have suggested that the heavy reliance

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on the acetylene reduction assay may have compromised accuracy on N fixation heritability studies. Nonetheless, their own conclusions regarding the possibility of enhanced N fixation through breeding in soybean have been positive (Herridge and Danso, 1995; Herridge and Rose, 2000).

Despite the promising diversity and heritability of N fixation, a major obstacle to successful breeding is the complexity of the host plant–rhizobia symbiosis and the difficulty of identifying improved germplasm through conventional screening. Furthermore, modern legume breeding tends to trail other major crops in the majority of agronomically required traits, inevitably pushing nitrogen fixation down the list of priorities and discouraging the introgression of landraces that might destabilize other essential traits.

106.2 POTENTIAL TRAITS AND BREEDING STRATEGIES THAT CONFER ENHANCED FIXATION

Effective advances in N fixation breeding may be realized through a range of approaches.

106.2.1 Host–Symbiont Compatibility

The overall productivity of nitrogen fixation depends largely upon the compatibility of both the legume plant and rhizobia strain, making cultivar–strain interaction an important consideration for breeding. The synergistic relationship between the host and rhizobia strains can be one of the most crucial factors for achieving symbiotic potential (Abi-Ghanem et al., 2011; Santalla et al., 2001).

In their review, Herridge and Rose (2000) suggest two breeding strategies to deal with host–strain specificity, namely, target cultivars that form effective symbioses with a broad range of rhizobia (often referred to as *promiscuous* nodulation) or target cultivars that preferentially nodulate with more productive rhizobia and exclude the less productive lines (selective nodulation). Ultimately, screening for productive symbioses across cultivars needs to take field variation into account as indigenous rhizobia populations can often competitively nodulate legumes but fix nitrogen poorly, potentially confounding the results and frustrating breeding efforts.

Evaluating the compatibility of subterranean clover (*Trifolium subterraneum*) genotypes, Drew and Ballard (2010) inoculated 49 clover genotypes with 4 rhizobial strains isolated from soil extracts. The symbiotic performance (SP) of each cultivar and strain was determined by calculating plant growth as a percentage of shoot dry matter when inoculated with the recommended commercial inoculant. Their findings indicated a significant effect of both cultivar and rhizobia strain on plant growth. Broad variation of SP was also noted across genotypes when inoculated with the commercial strain (WSM1325), with some varieties showing less than half the shoot dry weight of other varieties, indicating the scale of impact that inappropriate inoculation has on N fixation. For further insight, a low SP genotype (cv. Clare) and a high SP genotype (cv. Campeda) were selected based on their comparable optimum dry weight. Both genotypes were inoculated with soil extracts that contained a diverse range of different rhizobia strains. The SP of Campeda across all rhizobia strains was on average 29% higher than that of Clare. Moreover, nodules were generally more efficient at fixing nitrogen in Campeda than Clare (inferred from leghemoglobin being present in nodules). Clare's poor performance was attributed to disruptions at multiple points in the nodulation process as shown by the lower number and less effective nodules. Drew and Ballard (2010) concluded that a wider compatibility range accounted for the superior SP observed, though the authors postulated that the greater SP in Campeda could imply that this genotype had an additional ability to discriminate against less effective rhizobia.

Investigating the cultivar–strain interaction in field pea, Martensson and Rydberg (1996) tested the interaction between 16 commonly grown European cultivars with 7 rhizobia strains. Results were collected at early and advanced plant maturity to differentiate between early symbiotic interactions and nitrogen uptake generally. Their results indicated no significant interaction in early stages of symbiosis initiation, highlighting that early nodulation does not necessarily imply greater N uptake or dry matter accumulation. Interestingly, results collected at advanced maturity showed that from 16 cultivars, Maro, Maxi, and Minnesota exhibited marked cultivar–strain interactions where N uptake ranged from moderately above average to well below average, whereas cultivars Bodil, Bohatyr, Filby, and Imposant showed no variation with moderate to good performance across the board, suggesting two possible N fixation breeding targets. Commenting on the dilemma that this imposed on plant breeders, the authors suggested that the former target of cultivars which form productive symbioses with a narrow range of strains might be the most sensible target due to better marketability to growers.

106.2.2 Selective Nodulation

Glasshouse and field studies have thoroughly demonstrated the pervasiveness of host–strain specificity and the gains to be made with effective coupling of symbionts (Laguerre et al., 2007). However, indigenous rhizobial populations that can competitively nodulate crop legumes but inefficiently fix nitrogen can undermine the benefits of symbiotic compatibility (Denton et al., 2002). Yates et al. (2008) demonstrated the selective nodulation ability of *Trifolium purpureum* and *T. polymorphum*, where both hosts were nodulated with an

effective and an ineffective rhizobia strain. Both hosts nodulated solely with the effective strain even when outnumbered a hundredfold.

It has been suggested that to counteract *free-riding* rhizobia, certain legumes have evolved the ability to impose sanctions on ineffective nitrogen fixers by preferentially supplying nodules with more productive rhizobia or blocking infection altogether (Denison 2000; Yates et al., 2008). However, the prevalence of nitrogenous fertilizers in modern agriculture and breeding programs may have had a deleterious impact on modern cultivars to discriminate between effective and ineffective rhizobia strains. In a field study of soybean cultivars ranging from 1937 to 2001, cultivars were inoculated with fixing and nonfixing rhizobial strains. Kiers et al. (2007) noted a clear trend of newer cultivars nodulating with ineffective strains more commonly than older cultivars, suggesting that contemporary breeding efforts in soybean may be having a retrograde effect on the efficacy of N fixation symbiosis. This finding corroborated the observations made by Van Kessel and Hartley (2000), who reviewed 362 studies of soybean and found a significant drop-off in N fixation according to studies conducted post-1985.

106.2.3 Nitrate-Tolerant Fixation

The inhibitory effect of nitrate on legume nitrogen fixation is a well-documented phenomenon (Voisin et al., 2002; Glyan'ko et al., 2009). A common explanation is that the carbon demand of nodulation exceeds the assimilation costs of mineral nitrogen, culminating in suppressed nodulation (Glyanko et al., 2009]. Nevertheless, the biochemical background to nitrate suppression of nodulation is complex. It cannot be entirely explained as an energy regulation decision by the plant, as it is specifically nitrate that poses the most severe reduction compared to other mineral nitrogen forms such as ammonia (Gibson and Nutman, 1960; Bollman and Vessey, 2006). Nitrate-tolerant symbiosis has been regarded as one of the most important targets for fixation breeding, as it would greatly increase the nitrogen benefits of legumes in high-fertility environments (Evans, 1982).

Two general strategies for targeting nitrate-tolerant symbioses are to select for natural variation within a population or through mutants carrying super- or hypernodulating genes. Looking to develop nitrate-tolerant symbiosis through natural variation, Herridge and Rose (2000) conducted a nitrate-tolerant breeding program of soybean by introducing 32 genotypes that displayed superior nodulation into a mainstream breeding program. Genetic variation for N fixation was first determined through glasshouse screening in low N sand-filled pots. Once selections were made, two subsequent field trials conducted on high nitrate soils in Australia tested the lines for sustained nodulation in response to soil nitrate. Korean cultivars in particular were observed to possess notable nitrate tolerance and nitrogen fixation activity in the field conditions. Postharvest soil tests revealed that on average 34 kg/ha nitrogen was conserved where high N-fixing Korean cultivars were present when compared to the commercial cultivar Bragg. Despite the gain in nitrogen, seed yield for the high N fixers was on average 30% below mainstream cultivars due to poor agronomic features. Nevertheless, correlation matrices suggested that the traits associated with yield loss were independent of the high N-fixing ability, leading to the conclusion that the Korean cultivars could be effective donor parents (Herridge and Rose, 2000). Four high-fixing Korean cultivars [K464, K466, K468, and K469] were subsequently hybridized with four high-yielding cultivars [Valder, Reynolds, Forrest, and Bossier]. From these combinations, 849 F_2 progeny were tested for N uptake, with results suggesting promising segregation for fixation. Single seed descent was used to generate F_5 and F_6 populations to be tested once again in the field for nitrate tolerance. Although these populations produced lines that yielded above some mainstream cultivars, such as Valiant, unfortunately no significant symbiotic advantage could be attributed to lines produced from the N fixation breeding program. Furthermore, the lack of disease resistance necessitated that lines produced from this program be shelved. Nevertheless, the program succeeded in identifying novel germplasm, and the Korean cultivars may well have delivered other benefits to the program such as seed protein content (Herridge and Rose, 2000).

106.2.4 Supernodulation

An intuitive strategy to enhance nitrogen fixation is to increase nodulation: the sites of dinitrogen reduction. The establishment of root nodules is facilitated by a complex signaling process between rhizobia and plant root hairs at the extremities of the root system. The host plant exudes secondary metabolites [flavonoids; see Chapter 50] that attract rhizobia and activate bacterial *nod* genes, eliciting the production of lipochitooligosaccharides also known as Nod factors (see Chapter 51). These factors induce the plant root to allow the infection of rhizobia to commence and ultimately cause the nodule structure to form (Franche et al., 2009; Mortier et al., 2011; see Chapter 59). The specificity between legume species and different rhizobial strains is largely accounted for by the structure of these signaling compounds (Ferguson et al., 2010).

After nodule formation, the production of ammonia from dinitrogen reduction requires sucrose and dicarboxylic acids for energy. This energy is delivered by the photosynthetic activity of the plant shoot (Caetano, 1997). However, the amount of energy partitioned to nodulation is carefully controlled through an autoregulation system similar to other shoot-to-root signaling pathways (Novak et al., 2009). Typically, when nodulation has reached a determined optimum, a substance termed as *Q* [most likely a CLAVATA3/ESR

[CLE]-related peptide] is synthesized by the root and is translocated to the shoot where it is transformed into a shoot-derived inhibitor (SDI) (Okamoto et al., 2009). The SDI is translocated back to the root system and halts the nodulation process (Li et al., 2009). A key gene that controls this pathway in the model species *Lotus japonicus* is *HAR1*, and its product has been identified as a receptor kinase (Krusell et al., 2002). Variants of this gene have been isolated in a number of legume species including soybean (Mortier et al., 2011), pea (Sagan and Duc, 1996), and *M. truncatula* (Searle et al., 2003; Schnabel et al., 2005).

Supernodulation [SN] is caused by a mutation that disrupts the autoregulatory signaling pathways to the root system and encourages an abnormal amount of nodules to form (Li et al., 2009). Generally, an overabundance of nodules has a deleterious effect on plant competitiveness as the increased nodulation demands an excessive carbohydrate supply (Voison et al., 2007). Sidorova et al. (2005) found the *nod4* SN inducing gene in field pea (*Pisum sativum*) to drastically limit plant growth and productivity while also reducing N fixation efficiency despite the large increase in nodulation. Attempting to remedy this effect, the SN mutant was crossed with three conventional cultivars (Torsdag, Truzhenik, and Falensky), and progeny were recurrently selected to the F_6 generation. The conventional cultivars were selected based on their difference in N fixation ability, with Torsdag and Falensky being efficient N fixers and Truzhenik an inefficient fixer. Their findings showed considerable alleviation of the SN effect at the F_6 generation on plant growth productivity while maintaining superior levels of nodulation and nitrogenase activity when compared to the wild-type cultivars. Nevertheless, all of the cultivar–mutant crosses incurred yield loss and growth reduction to some extent with the introduction of *nod4* when compared to the conventional parent. Despite this result, a strong cultivar effect was observed on plant yield and growth, leading to the conclusion that N fixation and photosynthetic ability of the background genotype is a critical consideration when incorporating SN mutations in a breeding program and appropriate genetic background could ultimately justify wider use of SN in breeding programs (Sidorova et al., 2005).

Keeping in mind the carbon demand that SN imposes on the plant, Novak et al. (2009) crossed an SN pea mutant with high-biomass forage pea cultivars, Arvika and Zhodino E9000, in an effort to balance the effect of excessive nodulation with plant ideotypes that conduct higher levels of photosynthesis. Similar to other parallel studies, growth deprivation was noted in the majority of recombinant lines when the SN mutation was introgressed into the wild-type cultivars. Although five lines carrying the SN mutation were found to exceed their forage-type parent in some growth characteristics, this did not translate to a completely successful counteractive of the SN trait (Novak et al., 2009). Nevertheless, the experiment showed that one recombinant line derived from forage cultivar Zhodino E900 possessed the ability to modify the SN mutation so that the number, size, and efficiency of nodules could be brought closer to the wild-type nodulation pattern, though their study stopped short of actually showing the beneficial effect of this modification. The authors suggested that SN modification was an additional counteractive strategy to previous attempts such as mutations to nodule number loci (Sagan and Duc, 1996) and suppressor mutations to the SN allele (Murray et al., 2006).

Theoretically, the seed and biomass yield reduction caused by SN may potentially be overlooked in forage systems where nitrogen nutrition is the primary target, as suggested by Novak et al. (2009). Nonetheless, these circumstances remain in the minority for legume breeding. Ultimately, incorporation of SN mutants into a crop breeding system should be accompanied by traits that can alleviate the growth and yield reduction that SN imposes.

106.2.5 Molecular Breeding Techniques

Given the numerous genetic influences on nitrogen fixation, molecular breeding techniques such as quantitative trait loci [QTL] mapping and marker-assisted selection can potentially help breeding programs that identify N fixation as a breeding target. However, the application of these technologies to N fixation traits remains in its infancy. Studying recombinant inbred line [RIL] populations from the model legume species *L. japonicus*, Tominaga et al. (2012) identified 34 QTLs associated with 10 traits relevant to nitrogen fixation and plant growth. Acetylene reduction activity, nodule number per plant, and stem length all displayed strong QTL correlations. Colocalization was discovered for a variety of trait QTLs and was additive with the sole exception of the acetylene activity per nodule weight trait. Promising discoveries were the detection of a nitrogen fixation regulating loci on chromosome 5 that promoted plant growth through the regulation of nodule development, the detection of an allele on chromosome 4 that influenced yield and plant growth via regulation of nitrogen, and a QTL on chromosome 3 that affected nodule number. As linkage maps and marker identification become available, the application of this knowledge to breeding for N fixation may help unravel the many interacting factors associated with N fixation (see Chapter 78).

Furthermore, utilizing a recently published integrated genetic linkage map of soybean, Nicolas et al. (2006) identified two genomic regions associated with nodule number and nodule dry weight in the F_2 progeny derived from Embrapa 20 × BRS 133, as well as another 13 significant associations between QTL and marker loci relevant to enhanced fixation. For the portion of the genome tested (21 SSR markers, approximately 10%), phenotypic variation

in nodule number and dry weight accounted through linked markers was 2–9% and 3–6%, respectively. This indicated that subsequent studies into the remaining genome will further augment the understanding of phenotypic variation for N fixation (Nicolas et al., 2006).

Working with seven cultivars of field pea (Ballet, Cameor, China, VavD265, K586, Sommette, Terese), Bourion et al. (2010) measured nitrogen acquisition structure as determined by root system size, prolonged nodulation period, and the effect these had on nitrogen nutrition. Significant genotypic variation was observed for both root structure and nodulation traits across all seven cultivars. Nodulation appeared to be particularly variable with certain growth stages showing 10-fold differences between genotypes. Broad-sense heritability was calculated showing that root and nodule traits were found to be highly heritable [0.88 and 0.77, respectively]. To investigate the genetic contribution further, a RIL population of Ballet × Cameor was genotyped based on the dissimilarity in root and nodule traits. 20 QTLs were identified to have an effect, 3 were identified that were associated with the proportion of nitrogen derived through fixation [%Ndfa], 7 were linked to N acquisition, and a further 10 were identified for nitrogen fixation efficiency traits. Of these 10, 5 were located within the same genomic region and correlated with 9–21% of the phenotypic variation. QTLs were also observed in four additional genomic regions contributing between 8% and 12% of the variation. Interestingly, cv. Cameor alleles contributed solely positive effect QTLs for fixation efficiency and solely negative effect for %Ndfa. All QTLs for %Ndfa corresponded to QTLs associated with root and nodule traits, indicating that targeting traits that promote root development prior to seed filling, when nitrogen fixation is most intense, would be an effective breeding target (Bourion et al., 2010).

However, the difficulty in interpreting and implementing large numbers of QTLs for complex traits into breeding programs is a well-recognized phenomenon (Mir et al., 2012). Given the complex of interacting factors that constitute N fixation symbiosis, molecular approaches such as QTL mapping for marker-assisted selection will present their own host of challenges.

106.3 CONCLUSION

The advantages of biological nitrogen fixation will increase as fertilizer costs and environmental concerns become more pressing. However, progress to date in breeding for these traits has been slow due to the trait complexity and competing breeding targets in legume programs. Nevertheless, continued research into Supernodulation, host–strain compatibility, and nitrate-tolerant symbiosis combined with advanced molecular approaches could lead to substantial increases nitrogen fixation within the farming system. However, it is likely that large-scale enhancement will only be delivered as N fixation becomes less of a research subject and more of a priority trait in breeding programs themselves.

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Chapter 107

LCO Applications Provide Improved Responses with Legumes and Nonlegumes

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107.1 INTRODUCTION

Signal molecules are essential for the penetration into plant roots by beneficial symbiotic microorganisms. Rhizobia are symbiotic nitrogen-fixing bacteria that penetrate legume roots via root hairs and enter the developing root nodules that contain the atmospheric nitrogen-fixing process. Lipochitooligosaccharides (LCOs) are Nod factor signal molecules produced by rhizobia after the legume plant exudes flavonoids that bind to rhizobia and turns on bacterial genes that synthesize the Nod factor molecules (Kamst et al., 1998; see Chapters 50, 51). The basic research and discovery of Nod factor structures (Broughton et al., 1997; Lerouge et al., 1996; Stacey et al., 1992, 1994; D'Haeze and Holsters 2002; Ardourel et al., 1994), specific root hair receptor binding sights (Limpens et al., 2003; see Chapter 51), and plant gene signaling cascade (Gough and Cullimore, 2011; see Chapter 59) leading to rhizobial root hair infection and nodule morphogenesis (Oldroyd and Downie, 2008) have been extensively reported.

Arbuscular mycorrhizae also produce LCOs known as Myc factor signal molecules (Maillet et al., 2011) that act in a similar manner allowing fungal penetration into plant root cells. More than sixty percent of land plants form this symbiotic relationship allowing the fungal mycelium to scavenge for the plant soil nutrients, particularly phosphorus, nitrogen, and sulfur (Harrison, 2005; see also Chapter 108).

Applied research investigating LCO direct plant growth responses in either the greenhouse or field trials has been far less studied and will be reported in this review.

Formulations of Nod factors combined with the rhizobia for the specific legume host, or Nod factors alone, provide several plant growth benefits when applied to seed. The Nod factor of *Bradyrhizobium japonicum* enhances the germination of important agronomic crops, to include legumes [soybean (*Glycine max*) and beans (*Phaseolus vulgaris)*] and nonlegumes (corn (*Zea mays*), rice (*Oryza sativa*), sugar beets (*Beta vulgaris*), and cotton *(Gossypium hirsutum*)] in greenhouse and field conditions (Prithiviraj et al., 2003; Smith et al., 2005). The Nod factor of *Rhizobium leguminosarum* biovar *viciae* increases germination of both peas (*Pisum sativum*) and hairy vetch (*Vicia villosa*) (Kidaj et al., 2011]. Enhanced legume nodulation with Nod factor seed treatments has been reported with *P. sativum* and *V. villosa*

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[Kidaj et al., 2011], *Medicago truncatula* (Macchiavelli and Brelles-Marino, 2004), and *Trifolium pratense* (Maj et al., 2009). Nod factors and Myc factors are active molecules which also increase mycorrhizal root colonization and stimulate lateral root development (Olah et al., 2005). Evidence of cross talk between the two molecules is suggested by these findings. These two signal molecules produced by either a bacterium (rhizobia providing Nod factors) or a fungus (mycorrhizae producing Myc factors) each have their specific LysM receptor-like kinases on the root that activates a common symbiotic plant gene pathway (Gherbi et al., 2008; known as CSSP, CSP or SYM; see Chapters 42, 54, 55, 108, 110). Downstream genes beyond the common symbiotic pathway provide for rhizobial nodulation or Mycorrhizal root penetration (see Chapter 59).

Nod factors have also been reported to provide improved early growth of both legumes and nonlegumes (Souleimanov et al., 2002; Prithiviraj et al., 2003; Kidaj et al., 2012; Maj et al., 2008]. Growth and plant vigor are most likely responses resulting from more specific Nod factor effects such as enhanced germination, lateral root development, and nutrient supply.

Further LCO plant responses have been reported to include overcoming the stressful effect of low pH on soybean root hair curling with higher levels of LCOs (Miransari et al., 2006), foliar applications of LCOs on soybeans, and corn enhancing photosynthesis (Khan et al., 2008; Smith et al., 2007), plus foliar LCO application with tomatoes increasing both flowering and fruit production (Chen et al., 2007).

The plant benefits of LCOs clearly extend beyond improved nodulation, to include various benefits from seed, in-furrow, or foliar applications.

107.2 METHODS

107.2.1 Lipochitooligosaccharide Formulations

Liquid LCO formulations (Nod factors and Myc factors) were prepared for application studies and commonly analyzed via HPLC and mass spectrum analysis. LCO preparations were formulated by either preincubating a rhizobial Nod gene activating flavonoid (see Chapter 50) (specific molecules are required for the various rhizobial species) with the rhizobium or providing precise concentrations of LCO via direct addition of a purified LCO molecule. Pure LCOs in reported studies by Smith S, Habib A, Kang Y, Leggett M, Diaz-Zorita M, Novozymes Biologicals, Inc., Salem, VA, unpublished results, were those with structures representing soybean or pea LCOs. Preincubation preparations with *B. japonicum* included genistein, a Nod gene inducer, at concentrations of 5 to 20 µg with 3-day-old *B. japonicum* cultures and incubation for two additional days (Zhang and Smith, 1995).

107.2.2 Root Hair Deformation Assays

Root hair deformation (RHD) is the first plant physical response to LCO molecules (see Chapters 41, 57). A semi-quantitative RHD assay for hairy vetch roots was utilized by replacing plant growth medium with a medium containing Nod factor within Fahraeus slides followed by incubation in Petri dishes at 22 °C for 3 hours. Microscopic examination of deformed root hairs was carried out, and deformation was rated as 0, 1, or 2 corresponding to 0–20%, 20–60%, and greater than 60% deformed root hairs (Heidstra et al., 1994). RHD was also evaluated utilizing 15-cm-long root segments of 5-day-old siratro (*Macroptilium atropurpureum)* roots. Root segments were soaked in 5 ml of a 10^{-8} M LCO solution in glass test tubes for 6 hours. Then the root segments were colored by adding 100 µl of 1% Congo red dye for 15 minutes. Root segments were observed for RHD under a compound microscope (Smith S, Habib A, Kang Y, Leggett M, Diaz-Zorita M, Novozymes Biologicals, Inc, Salem, VA, unpublished results).

107.2.3 Seed Germination Assays

Procedures for evaluating the effects of LCO on seed germination are provided by Kidaj et al. (2012). Pea and vetch seeds were surface sterilized, soaked for 30 minutes in serially diluted LCO solutions (from 10^{-10} to 10^{-12} M). Seeds were transferred to Fahraeus agar medium (10 seeds per container) and germinated in darkness at 28^0C. Germination was observed after 3 and 4 days for pea and 4 and 5 days for vetch. Prithiviraj et al. (2003) evaluated the activity of LCO on germination of *Z. mays, B. vulgaris, G. max, G. hirsutum, and P. vulgaris* in a greenhouse maintained at 25 $\pm 2^0$C. Twenty ml of a Nod factor preparation at concentrations of 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, and 10^{-11} M was irrigated into the pots and percent emergence determined for each concentration after 3 days.

107.2.4 Nodulation Assays

Procedures evaluating nodulation enhancement with the addition of LCOs have been reported in several previous studies with peas and vetch (Kiday et al., 2012), *M. truncatula* (Macchiavelli and Brelles-Morrino, 2004), and *T. pratense* (Maj et al., 2009).

107.2.5 Mycorrhization Assays

Nod factor effects on mycorrhization were evaluated (Olah et al., 2005) utilizing the model legume *M. truncatula* seedlings inoculated with the arbuscular mycorrhizal (AM) fungus *Gigaspora margarita* and then grown in sterile conditions in test tubes with and without Nod factors in the

medium. Nod factors from *Sinorhizobium meliloti* were used at 10^{-9} M and grown for 6 weeks under growth chamber conditions. Plant roots were stained and microscopically screened for mycorrhizal infection.

107.2.6 Root Growth Evaluations

Procedures for evaluating the effects of both Nod factors and Myc factors on lateral root growth and their interactions with auxin are provided by Olah et al. (2005). Root enhancement was also evaluated with LCO on corn seeds (Smith S, Habib A, Kang Y, Leggett M, Diaz-Zorita M, Novozymes Biologicals, Inc, Salem VA, unpublished results). Seeds were treated with 10^{-8} M Nod factor solution, placed on moist germination paper in the dark for germination. After 7 days, several root parameters were measured using the WinRhizo^{TM} Scanner.

107.2.7 Plant Growth Evaluations

The effects of LCO on early plant growth has been evaluated and described in several studies. Pea and vetch were evaluated in growth chamber studies using seeds soaked in serially diluted LCOs (from 10^{-10} to 10^{-12}M) and grown for 6 weeks. Further pot studies with two arable soils evaluated early growth for 6 weeks outdoors under a shelter (Kidaj et al., 2012). Seeds of *G. max, Z. mays, B. vulgaris, G. hirsutum, Cucumis sativus*, and *P. vulgaris* were grown in pots within growth chambers (Prithiviraj et al., 2003). Pots were irrigated with 25 ml of LCO solution (10^{-6} to 10^{-11}M) and harvested after 15 days with early growth parameters measured. Further greenhouse studies with corn seeds treated with 10^{-9}M LCO and grown for 3 weeks, cotton seeds treated with 10^{-8}M LCO and grown for 6 weeks, and LCO-treated soybean seeds (10^{-8}M LCO) harvested after 5 weeks evaluated early plant growth parameters (Smith S, Habib A, Kang Y, Leggett M, Diaz-Zorita M , Novozymes Biologicals, Inc, Salem, VA, unpublished results).

Foliar applications of soybean LCO (Nod BjV(C18:1, MeFuc)] were evaluated at concentrations of 10^{-6}, 10^{-8}, and 10^{-10}M on corn and soybeans, and the photosynthetic rate 3 days after application was determined (Kahn et al., 2008). The foliar application of LCO was further evaluated with greenhouse tomatoes (*Solanum lycopersicum*) by spraying the leaves twice with a 5 ml water solution containing LCO concentration of 10, 50, or 100 ng per plant (Chen et al., 2007). The first application was at early flowering stage and the second application 2 weeks later. Field studies were also conducted (Chen et al., 2007) with the LCO dosage ranging from 1 to 1000 ng (1.4×10^{-10} to 1.4×10^{-7}M in a 5 ml solution) per plant once at the onset of flowering and/or fruit set stages. Leaf parameters including chlorophyll content (estimated via a SPAD chlorophyll meter), leaf carbohydrate content by colorimetric assay (Dubois et al., 1956), and leaf NO_3-N were measured from the third fully opened soybean leaf from the top at two weeks after foliar LCO application (Smith S, Habib A, Kang Y, Leggett M, Diaz-Zorita M, Novozymes Biologicals, Inc, Salem, VA, unpublished results).

Nod factor field trials with legumes (soybean and peas) and nonlegumes (corn, cotton, and wheat) were conducted through University and third-party research contractors (Smith and Habib, 2009). Soybean (LCO BjV (C18:1,MeFuc)] and pea [LCO Rl V (C18:1)] Nod factors were prepared in liquid formulations at concentrations ranging from 10^{-7} to 10^{-9}M. Seed applications ranged from 5 to 10 ml kg^{-1}, in-furrow applied at 0.1 ml m^{-1} of row, and foliar applications were applied at early growth stage and in combination with glyphosate herbicides. Small field plots had 4–6 replications and were in a randomized complete block design, while large strip plots with 4 replications were randomized. Multiple-site analyses from North and South America and from multiple years were combined and evaluated. Probability that the mean percent yield difference between the treated and control was greater than 0 was tested using the Wilcoxon signed-rank test, JMP10 software SAS Institute, Inc., 2012.

107.3 RESULTS

Direct and indirect plant responses with the application of Nod factors (LCOs from rhizobia) have been widely reported with both legumes and nonlegumes.

107.4 ROOT HAIR DEFORMATION

The first direct effect of Nod factors is the RHD response, which requires 5 to 10 minutes of Nod factor—root hair interaction. The first deformation with *Vicia sativa* was visible within 1 hour, and after 3 hours, about 80% of the root hairs in a small susceptible zone of the root were deformed (Heidstra et al., 1994). Suboptimal temperatures (15 to 17.5° C) delayed the rhizobial Nod factor production because the Nod gene inducing molecule (flavonoids) were not released from the roots (Zhang and Smith, 1995). The time for soybean RHD to begin under these conditions was shortened by inoculating with a *B. japonicum* culture preincubated with genistein to cause Nod factor production (Zhang and Smith, 1995).

107.5 GERMINATION AND EMERGENCE

Seeds treated with Nod factors have provided enhanced seed germination and emergence (Smith et al., 2005). Growth chamber studies with soybean Nod factors (LCO

Table 107.1 Effect of the Nod factor Nod BjV(C18:1, MeFuc) on seedling emergence of four crop species under field conditions measured 10 days after planting (adapted from Prithiviraj et al., 2003)

Treatment	*Zea mays*	*Gossypium* Hirsutum	*Beta vulgaris*	*Glycine max*
	% Emergence			
Control	41.6b	6.6b	26.6b	16.6c
LCO				
10^{-5}M	80.0a	16.6b	28.3b	26.6bc
10^{-7}M	60.6b	60.0a	46.6a	33.3b
10^{-9}M	53.3b	23.3b	38.3ab	63.3a

Within Each Column (Crop), Numbers Followed by the Same Letter Do Not Differ Significantly by an ANOVA-Protected LSD Test at P<0.05

BjV(C18:1,MeFuc)], inoculated on various economically important plants belonging to diverse botanical families, provided significantly improved emergence with corn (*Z. mays*), sugar beets (*B. vulgaris*), soybean (*G. max*), cotton (*G. hirsutum*), and beans (*P. vulgaris*) (Prithiviraj et al., 2003). Further studies with four crop species under field conditions also provided significantly improved emergence when treated with Nod factor concentrations of 10^{-5}, 10^{-7}, and 10^{-9}M (Table 107.1) (Prithiviraj et al., 2003). Growth chamber studies with extremely low amounts of *R. leguminosarum* bv. *viciae* Nod factors provided significant percent germination increases for pea (control without Nod factors $87\% \pm 13$, 10^{-10} M Nod factors $93\% \pm 11$, 10^{-11} M Nod factors $97\% \pm 13$, and 10^{-12} M Nod factors $96\% \pm 9$) and vetch (control $70\% \pm 9$, 10^{-10} M Nod factors $82\% \pm 11$, 10^{-11} M Nod factors $78\% \pm 6$, and 10^{-12} M Nod factors $73\% \pm 5$) (Kidaj et al., 2012).

107.6 NODULATION

Nodulation occurs when combining an infective rhizobium with its legume host. Studies have reported either earlier or increased nodulation when supplementing the rhizobial inoculum with the required Nod factor. The model legume *M. truncatula* inoculated with *S. meliloti* and the *S. meliloti* Nod factor significantly increased nodulation 15 days after inoculation with Nod factor concentrations of 10^{-8} M (2.20 ± 0.36 nodules plant^{-1}) and 10^{-9} M (2.32 ± 0.38 nodules plant^{-1}), but not with the rate of 10^{-7} M (1.30 ± 0.25 nodules plant^{-1}) (Macchiavelli et al., 2004]. Nodulation was also enhanced with pea seeds treated with *R. leguminosarum* bv. *viciae* Nod factors (control without Nod factors 61.6 ± 0.6 nodules plant^{-1}) as compared to Nod factor-treated seeds (102.6 ± 23.6 nodules plant^{-1}) (Kidaj et al., 2012). Hairy vetch seeds treated in a similar manner with Nod factors provided 87.6 ± 64 nodules plant^{-1} without Nod factors and 107.6 ± 10 nodules plant^{-1} with Nod factors. Nod factor treatments of clover seeds significantly (P<0.05) enhanced clover nodulation and growth of plants (Maj et al., 2009). Soybean seed inoculated with *B. japonicum* cultures preincubated with genistein, causing Nod factor production by the bacteria, increased nodulation at the suboptimal root zone temperatures (RZT) of 15.0 and 17.7 °C, but not at the optimal RZT of 25 °C (Zhang and Smith, 1995).

107.7 Nod FACTOR: Myc FACTOR CROSS TALK

Nod factors and Myc factors utilize a common plant gene pathway before their specific activation leading to nodulation or mycorrhizal root infection, respectively (Olah et al., 2005). Cross talk between these two LCO molecules was illustrated by the observation that Nod factors from *S. meliloti* increased mycorrhizal colonization in *M. truncatula* (Olah et al., 2005).

107.8 ENHANCED ROOT DEVELOPMENT

Further plant responses to LCO include enhanced root development. Both Nod factors and Myc factors provide this response. *S. meliloti* Nod factors very significantly ($P < 0.01$) stimulated *M. truncatula* lateral root formation at concentrations of 10^{-7}, 10^{-8}, and 10^{-9} M. The increase was in the range of 35–40% (Olah et al., 2005). Nonsulfated Myc factors elicited root-branching stimulation (RBS) at concentrations lower than 10 nM. Sulfated Myc factors and the 1:1 mixture with nonsulfated Myc factors elicited RBS at concentrations lower than 0.01 nM. Both sulfated and nonsulfated Myc factors were thus extremely active with RBS, but sulfated molecules were about 100-fold more active (Maillet et al., 2011). Mycorrhizal signal molecules also stimulated root development and plant growth in

nonlegumes (Mukherjee and Ane, 2011). Various root parameters of corn and wheat treated with Nod factors were evaluated with 7-day-old seedlings and analyzed with the WinRhizo™ Root Scanner (Smith and Habib, 2009). Significant ($P < 0.05$) increases in root length (corn, control 40.7 cm vs. 10^{-8} M Nod factor 73.2 cm; wheat, control 18.4 cm vs. 10^{-8} M Nod factor 21.2 cm), root surface area (corn, control 10.5 cm^2 vs. 10^{-8} M Nod factor 19.4 cm^2; wheat, 3.6 cm^2 vs. 10^{-8} M Nod factor 4.0 cm^2), and root volume (corn, 0.2 cm^3 vs. 10^{-8} M Nod factor 0.4 cm^3) were observed.

107.9 EARLY GROWTH

Enhanced early plant growth, following seed treatment of corn with the Nod factor from *B. japonicum*, included shoot dry weight per plant and leaf area over a wide Nod factor application range. Shoot dry weight of the control (30.1 mg $plant^{-1}$) was not significantly different from the highest Nod factor concentration of 10^{-5} M (41.6 mg $plant^{-1}$) nor the lowest rate of 10^{-11}M (38.0 mg $plant^{-1}$). However, all rates in between provided significant differences (P<0.05) in mg $plant^{-1}$ compared with untreated control (10^{-6} M 51.4 $plant^{-1}$, 10^{-7} M 51.6 $plant^{-1}$, 10^{-8} M 66.2 $plant^{-1}$, 10^{-9} M 46.8 $plant^{-1}$, and 10^{-10} M 51.4 $plant^{-1}$) (Prithiviraj et al., 2003). The authors found similar results with leaf area (cm^2) comparing the untreated control (7.63 cm^2 $plant^{-1}$) to Nod factor treatments of 10^{-5} M (11.7 cm^2 $plant^{-1}$), 10^{-6} M (18.2 cm^2 $plant^{-1}$), 10^{-7} M (15.7 cm^2 $plant^{-1}$), 10^{-8} M (19.3 cm^2 $plant^{-1}$), 10^{-9} M (17.0 cm^2 $plant^{-1}$), 10^{-10} M (13.9 cm^2 $plant^{-1}$), and nonsignificance at 10^{-11} M (8.51 cm^2 $plant^{-1}$). Early plant growth enhancement of corn and cotton was observed following the seed treatment with Nod factors. Corn seeds treated with Nod factors at a 10^{-9} M rate provided significant (LSD $P < 0.05$) increases in dry matter production with both root (untreated 0.32 g $plant^{-1}$ vs. Nod factor 0.35 g $plant^{-1}$) and shoot (untreated 0.57 g $plant^{-1}$ vs. Nod factor 0.64 g $plant^{-1}$). Cotton seeds treated with Nod factors also obtained significant (LSD P<0.05) increases in root fresh weight (untreated 0.74 g $plant^{-1}$ vs. Nod factor 1.69 g $plant^{-1}$) and shoot fresh weight (untreated 4.95 g $plant^{-1}$ vs. Nod factor 8.73 g $plant^{-1}$) (Smith and Habib, 2009).

107.10 FOLIAR APPLICATION

Nod factors applied as a foliar treatment have also provided increased plant growth responses (Smith et al., 2005). The foliar application of Nod factor (Nod BjV(C18:1 MeFuc)] enhanced ($P < 0.05$) the photosynthetic rate of corn. The increases were 36%, 23%, and 12% for 10^{-6}, 10^{-8}, and 10^{-10} M treated plants, respectively (Khan et al., 2008). This study indicates that Nod factors can enhance the photosynthetic rate and growth of corn. Foliar treatment of 10^{-8} M Nod factor with greenhouse soybeans at the v3 growth stage (Fehr and Caviness, 1977) provided increases in various plant parameters. Leaf chlorophyll concentration 14 days after application was 30.1 in untreated plants compared to 33.3 (Minolta SPAD meter units) with plants treated with Nod factors. Total leaf sugar content 2 weeks after application was 909.3 µg in untreated plants compared to 1362.2 µg in plants treated with Nod factors. Leaf nitrate concentration 2 weeks after application was also increased from 2.96 mg in untreated plants to 4.26 mg in plants treated with Nod factors (Smith S, Habib A, Kang Y, Leggett M, Diaz-Zorita M, Novozymes Biologicals, Inc, Salem, VA, unpublished results).

107.11 YIELD ENHANCEMENT

Yield enhancement with seed applications of Nod factors was first reported for soybeans (Smith et al., 2004). Two field trials were established: a virgin soybean site without *B. japonicum* and a site previously planted to soybeans with indigenous *B. japonicum*. The Nod factor treatment provided a nonsignificant response at the virgin site, while *B. japonicum* alone and the rhizobium combined with Nod factors provided a significant ($P < 0.10$) yield enhancement. At the indigenous site, the Nod factor treatment, the *B. japonicum* alone, and the *B. japonicum* plus Nod factors provided increased seed yield ($P < 0.10$). In both trials, the largest yield was obtained with the *B. japonicum* plus Nod factor treatment (Table 107.2).

Multiple-site and multiple-year field analyses in North and South America show that treatments containing Nod factors from *B. japonicum* provided soybean grain yield increases compared to untreated control (Table 107.3) (Smith S, Habib A, Kang Y, Leggett M, Diaz-Zorita M, Novozymes Biologicals, Inc, Salem, VA, unpublished results). The largest yield increase occurred in Argentina

Table 107.2 Soybean grain yield performance with Nod factors, applied with and without *Bradyrhizobium japonicum* seed inoculation, on virgin soil and soil with indigenous *B. japonicum* (Adapted from Smith et al., 2004)

	Yield (kg ha^{-1})	
	Virgin Soil	B.j. Indigenous Soil
Control	3335	2499
B. japonicum	3588	2721
Nod factors	3487	2916
B.j. + Nod factors	3635	2923
LSD 10%	235	134
CV%	5.6	4.0

Table 107.3 Grain yield response of legumes, grown in North and South America field trials, to seed treatment with rhizobial inoculum containing Nod factors

Crop	Country	Seasons (Harvest)	Number of Sites	Yield Increase (%)	Lower 95% CI	Upper 95% CI	Sites with Positive Response (%)	Probability that % Increase >0*
Soybean	Argentina	2002–2012	476	8.9	7.7	10.1	76.9	<0.0001
Soybean	Brazil	2007–2011	46	3.7	0.4	6.8	54.3	0.0150
Soybean	United States	2009–2011	529	4.1	3.3	4.9	65.4	<0.0001
Soybean	Canada	2000–2012	19	25.4	11.7	39.0	82.4	<0.0001
Pea	United States	2004–2011	70	15.1	8.8	21.3	77.1	<0.0001
Pea	Canada	2004–2006	21	8.9	2.6	15.2	80.8	<0.0001

CI = Confidence Interval

*Wilcoxon Signed-Rank Test

Table 107.4 Grain yield responses of nonlegumes (Corn and Cotton) in North and South America field trials with Nod factor-treated seeds

Crop	Country	Seasons (Harvest)	Number of Sites	Yield Increase (%)	Lower 95% CI	Upper 95% CI	Sites with Positive Response (%)	Probability that % Increase >0*
Corn	Argentina	2010–2012	41	2.1	0.0	4.2	61.0	0.0310
Corn	Brazil	2011–2012	43	2.9	0.4	5.4	65.1	0.0052
Corn	United States	2009–2011	60	2.1	0.6	3.7	66.7	0.0047
Cotton	United States	2007–2011	32	4.73	1.8	7.7	71.9	0.0022

CI = Confidence Interval

*Wilcoxon Signed-Rank Test

and Canada with increases of 8.9% and 25.3%, respectively. Yields greater than control were found in 14 of 17 trials (84.2%) in Canada and in 366 of 476 trials (82.4%) in Argentina. In the United States, treatments containing Nod factors were greater in 346 of 529 trials (65.4%) with an average of 4.3% yield increase. Yield increases in Brazil occurred in 25 of 36 trials (54.3%) with an average of 3.7% yield increase. Pea grain yield was also generally enhanced with treatments containing Nod factors from *R. leguminosarum* bv. *sativa*. In the United States, grain yield increases occurred in 54 of 70 trials (77.1%) with a 15.1% average yield increase. Canadian trials increased in 17 of 21 trials (80.8%) with an 8.9% yield enhancement.

Field trials were also conducted in North and South America with Nod factors as seed treatments on nonlegumes (corn and cotton) providing similar results in the United States, Argentina, and Brazil. Forty of 60 US corn trials (66.7%) increased yields providing an average of 2.1% yield enhancement (Table 107.4) [Smith S, Habib A, Kang Y, Leggett M, Diaz-Zorita M, Novozymes Biologicals, Inc, Salem, VA, unpublished results). Positive corn grain yield responses with Nod factors were found in 25 of 41 trials (61.0%) in Argentina enhancing yields by 2.1%, and in Brazil, 28 of 43 trials (65.1%) had positive results with a 2.9% average increase. Cotton trials in the United States responded in a similar manner with positive yield increases in 23 of 32 trials (71.9%) with an average increase of 1.8% (Table 107.4) [Smith S, Habib A, Kang Y, Leggett M, Diaz-Zorita M, Novozymes Biologicals, Inc, Salem, VA, unpublished results).

Studies with the foliar application of Nod factors also provided improved plant yield responses. Foliar application of *B. japonicum* Nod factors on greenhouse tomatoes increased flower numbers and early fruiting leading to increased tomato fruit yield (Chen et al., 2007). *B. japonicum*-extracted Nod factors foliarly applied to greenhouse tomatoes enhanced fruit weight when harvested 7 weeks after application (control 76.2 g plant^{-1} vs. Nod factor 10 ng 87.1 g plant^{-1}, 50 ng 104.3 g plant^{-1}, and 100 ng rate 88.0 g plant^{-1}) (Chen et al., 2007). Two foliar applications of Nod factors in field-grown tomatoes provided a significant enhancement ($P < 0.05$) in the number of fruit production (control 29.28 fruits plant^{-1} vs. a range of Nod factor rates: 1 ng 33.96 fruits plant^{-1}, 10 ng 42.50 fruits plant^{-1}, 100 ng 36.37 fruits plant^{-1}, and 1000 ng 35.00 fruits plant^{-1}) (Chen et al., 2007). Both fruit number per plant and fruit weight were increased by up to 29.4 and 29.8%, respectively (Chen et al., 2007). These studies indicate that

Table 107.5 Grain yield responses with Nod factor foliar applications on soybean and corn in North and South America field trials

Crop	Country	Seasons (Harvest)	Number of Sites	Yield Increase (%)	Lower 95% CI	Upper 95% CI	Sites with Positive Response (%)	Probability that % Increase >0*
Corn	United States	2007–2011	71	3.2	1.7	4.6	76.1	<0.0001
Corn	Argentina	2010–2012	40	2.1	−2.2	6.5	60.0	0.1437
Corn	Brazil	2010–2012	18	5.1	1.3	8.9	77.8	0.0061
Soybean	Canada	2008	6	2.4	−0.9	5.8	66.7	0.0469
Soybean	United States	2007–2011	109	2.7	1.6	3.8	66.1	<0.0001
Soybean	Argentina	2008–2012	85	3.9	0.66	7.1	51.8	0.0070
Soybean	Brazil	2010–2011	19	2.5	−1.4	6.5	63.2	0.1297

CI = Confidence Interval

*Wilcoxon Signed-Rank Test

a Nod factor foliar application to tomatoes in the range of 10 to 50 ng plant^{-1} can increase early flowering and fruiting, which results in improved fruit yields. Approximately 350 field trials in North and South America with the foliar application of Nod factors also provided a grain yield increase of soybeans and corn (Table 107.5) (Smith S, Habib A, Kang Y, Leggett M, Diaz-Zorita M, Novozymes Biologicals, Inc, Salem, VA, unpublished results). Foliar application of Nod factors increased yield ($P < 0.10$) of soybeans in North America (United States 2.7% and Canada 2.4%) and South America (Argentina 3.9% and Brazil 2.5%). Treatments were applied during early vegetative growth stage and in combination with a glyphosate herbicide, which provided a convenient method for application. The yield increases with Nod factors applied as a foliar treatment to corn were also evaluated in North and South America (Table 107.5). Corn grain yields in the United States were increased by an average of 3.2% and positive yield enhancements occurred in 76.1% of the trials. In Argentina, grain yields were increased an average of 2.1% with 60.0% of the trials providing a yield enhancement. Trials in Brazil provided a greater response with an average of 5.1% yield enhancement with 77.8% of the trials with increased yields.

107.12 DISCUSSION

Plant responses to LCO molecules, both Nod factors and Myc factors, go beyond the well-studied basic understanding of the specific receptors, common pathway of activated plant genes, rhizobial or mycorrhizal infection leading to nodulation, and arbuscule development followed by symbiotic nitrogen fixation and enhanced phosphorus uptake, respectively. Improved seed germination, greater mycorrhization, enhanced root development, better early plant growth, and higher yields frequently result from the LCO application on seeds, and also applications in-furrow, or as a foliar treatment. This review focuses on plant responses to Nod factors; however, Myc factor LCO molecules are also currently providing improved plant development (Olah et al., 2005).

The plant responses to the Nod factor signal molecules begin with RHD, which may be disrupted by environmental stress responses. The study of Zhang and Smith (1995) provides evidence that suboptimal temperatures (15 to 17.5 °C) decreased the release from the plant roots of the rhizobial Nod gene inducing molecules and thereby limited Nod factor production. This suboptimal temperature RHD response and the stress effects of low pH were improved by the addition of Nod factor molecules (Duzan et al., 2004).

The addition of Nod factors on the seed before planting improved the plant seed germination and emergence. The germination response was reported under growth chamber conditions with the application of the soybean Nod factor on important agronomic crops (corn, sugar beets, soybean, cotton, and beans) (Prithiviraj et al., 2003). The authors also found improved seedling emergence under field conditions with Nod factors applied to corn, soybean, cotton, and cucumbers.

Nodulation was the anticipated result of the Nod factor activation of plant genes leading to both rhizobial root hair infection and cortical cell morphogenesis into nodule formation. Supplementing Nod factors with the appropriate legume rhizobial host indeed did improve nodulation with *M. truncatula* (Macchiavelli et al., 2004], pea and vetch (Kidaj et al., 2012), clover (Maj et al., 2009), and soybean (Zhang and Smith, 1995). However, further plant growth parameters are enhanced with the addition of Nod factors. Enhanced root development is reported by the addition of either Nod factors or Myc factors (Olah et al., 2005; Maillet et al., 2011; Smith and Habib, 2009). This early plant root growth is likely the driving force to improved early plant growth leading to increased plant yields, plus the plants improved ability to withstand environmental stresses. Earlier and improved root

systems are likely to provide for better nodulation opportunities leading to enhanced nitrogen fixation and mycorrhization delivering increased phosphorus to the plant. Extending root systems further into the soil will provide the plant with more soil nutrients uptake and access to more soil water to mitigate abiotic stress (i.e., desiccation). Enhanced early plant growth occurred following Nod factor seed treatment with corn (Prithiviraj et al., 2003; Smith and Habib, 2009) and cotton (Smith and Habib, 2009).

Application of Nod factors to plant foliage appears independent from the plant responses to Nod factors delivered on or with the seed at planting. An increase in the photosynthetic rate (Smith et al., 2007; Khan et al., 2008) improved plant leaf parameters (chlorophyll, sugars, and nitrate (Smith S, Habib A, Kang Y, Leggett M, Diaz-Zorita M, Novozymes Biologicals, Inc, Salem, VA, unpublished results), and earlier flowering and fruiting (Chen et al., 2007) are reported after the foliar application of Nod factors. These foliar plant responses with Nod factor treatment are reported with legumes and nonlegumes, but the lack of understanding of molecular modes of action warrants further investigation.

Soybean grain yield enhancements with the seed application of Nod factors is reported when comparing sites with and without indigenous populations of *B. japonicum* (Smith S, Habib A, Kang Y, Leggett M, Diaz-Zorita M, Novozymes Biologicals, Inc, Salem VA, unpublished results). Additional multiple-year trials in both North and South America with legumes (soybeans, and peas – North America only) and nonlegumes (corn and cotton) with Nod factors applied on the seeds, in-furrow with the seed, or as foliar application have provided improved yields [Smith S, Habib A, Kang Y, Leggett M, Diaz-Zorita M, Novozymes Biologicals, Inc, Salem, VA, unpublished results). This indicates effective Nod factor plant growth effects under various soils, environmental, and crop management conditions.

Nod factors from different rhizobial genera (*B. japonicum* and *R. leguminosarum* bv. *sativa*) have demonstrated improved plant responses in growth chamber and field studies. With legumes, the appropriate host–rhizobial combinations were utilized, but with nonlegumes, Nod factors from different rhizobial genus may provide plant growth responses.

The direct and indirect plant growth responses with Nod factors have been reported over a broad application range (10^{-6} to 10^{-12} M) (Prithivaraj et al., 2003; Kahn et al, 2008; Chen et al., 2007; Kidaj et al., 2012; Olah, et al., 2005) indicating plant responses at very low amounts of applied molecules. However, the responses are generally reported to be most effective in the midrange from 10^{-7} to 10^{-9} M.

Additions of LCO signal molecules (Nod factors or Myc factors) have demonstrated the activation of plant genes providing for nodulation and mycorrhization, respectively (Gough and Cullimore, 2011). In addition, these signal molecules provide for various improved plant growth parameters. This demonstrates the agronomic value of these LCO molecules leading to generally better crop yields.

Detailed modes of action for the various reported LCO plant effects remain to be identified. However, seed-applied LCOs with both legumes and nonlegumes frequently provide for earlier and season-long enhanced root development in field studies. It is proposed that enhanced root development, providing the plant with better water and nutrient uptake supporting overall plant growth and yields, is a major contributor from the LCO mode of action. The mode of action for foliar LCO applications remains a subject for further research.

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Section 18

Nitrogen Fixation and Cereals

Chapter 108

The Quest for Biological Nitrogen Fixation in Cereals: A Perspective and Prospective

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108.1 INTRODUCTION

Recent advances in understanding symbiotic *Rhizobium*–legume and nonlegume interactions, actinorhizal–nonlegume interactions, and widespread arbuscular mycorrhizal (AM)–plant interactions at the molecular level, as described in various sections of this book, have stimulated the quest for biological nitrogen fixation (BNF) in cereals, such as rice. Moreover, the studies on the molecular genetics of the genes required for BNF in free-living diazotrophs such as *Azotobacter vinelandii*, described in this book, and methods to stably transform rice with heterologous genes have further stimulated this quest. The discovery of associative and endophytic diazotrophs in rice has stimulated research in this area, as described in this book as well. These advances will be discussed in this review. For other reviews on this topic, see Beatty and Good (2011) and Charpentier and Oldroyd (2010) and Chapters 5 and 109 of this book.

Rice is the most important staple food for over two billion people in Asia and for hundreds of millions in Africa and Latin America. To feed the ever-increasing populations of these regions, the world's annual rice production must increase from 560 million tons in the year 2000 to 760 million tons by 2020 (IRRI, 1993). If future increases in rice production have to come from the same or even reduced land area, rice productivity (yield ha-1) must be greatly increased to meet these goals (Ladha et al., 1997b). Nitrogen is the major nutrient limiting rice production. Rice requires 1 kg of nitrogen to produce 15–20 kg of grain (Ladha and Reddy, 2003).

Enhancing rice production from the present 8 to 12 ton per hectare by 2020 would require an increased application of 400 kg per hectare, doubling the amount of N fertilizer applied (Ladha and Reddy, 2003). The industrial production of nitrogen fertilizer comes at a cost of more than $100 billion per year and requires the use of large amount of fossil fuel, including petroleum, which, in turn, is highly vulnerable to political and economic fluctuations in the oil markets. Thus, fertilizer costs are high and this affects especially resource-poor farmers worldwide. In addition, the use of fertilizer has severe environmental impacts, due to runoff of excess nonassimilated nitrate, and concomitant eutrophication of rivers, lakes, and the ocean, as well as contamination of the drinking water. Moreover, carbon dioxide which is released during fossil fuel combustion to produce chemical fertilizer contributes to the greenhouse effect, as does the decomposition of nitrogen fertilizer, which releases nitrous oxide, itself about 292 times more active as a greenhouse gas than carbon dioxide (Ferguson et al, 2010; Crutzen et al., 2007). This obviates the need for alternative approaches, namely, BNF (see Chapter 109).

Diazotrophs in rice cultivation can be broadly grouped into two existing BNF systems, with the possibility of an additional two novel systems: (i) Indigenous BNF

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systems comprised of heterotrophic and phototrophic bacteria, endophytes, as well as native cyanobacteria; (ii) Exogenous BNF systems including *Azolla* harboring symbiotic N_2-fixing cyanobacteria and aquatic legumes like *Sesbania* and *Aeschynomene* forming symbioses with heterotrophic and phototrophic rhizobia (green manures). These systems need to be applied/inoculated into rice fields and involve additional labor, which is not preferred by farmers; (iii) Endogenous (*in planta*) BNF resulting from a rice plant transformed with the nitrogen fixation (*nif*) genes (Ladha and Reddy, 2003; Chapters 5 and 109); (iv) Symbiotic (nodular) endogenous BNF by rhizobia. The BNF systems 1, 3, and 4 will be discussed here. For further discussion, see Ladha and Reddy (2003).

Rice suffers from a mismatch of its N-demand and N supplied as fertilizer, resulting in a 50–70% loss of applied N fertilizer (see Chapter 5). Two basic approaches may be employed to solve this problem. One is to regulate the timing and level of N-application based on the need of the plant (see chapter by Kronzucker and Coskun (Chapter 5] and Beatty et al. [Chapter 109] of this book). The other is to increase the ability of the rice system to fix its own nitrogen. The latter approach is a long-term strategy, but it would have enormous environmental benefits while helping resource-poor farmers. Furthermore, farmers more easily adopt a (genetically modified) genotype or variety with useful traits than they do crop and soil management that are associated with additional costs (labor) (Ladha et al., 1997a). Recent advances in understanding the symbiotic Rhizobium–legume and AM–plant interactions at the molecular level, the structure and function of the nitrogen fixation (*nif*) genes, and the ability to introduce new genes into rice via transformation have created a new opportunity to incorporate BNF capability into rice.

108.2 PAST INVESTIGATIONS (PERSPECTIVE)

During a think-tank workshop organized by the International Rice Research Institute (IRRI) in 1992, the participants reaffirmed the existence of these possibilities and subsequently IRRI developed a New Frontier Project to coordinate a concerted international effort and constituted an International Rice Biological Nitrogen Fixation Working Group to review, to share research results and materials, and to catalyze research in this area (Ladha et al., 1997a). The second meeting of the Working Group took place in October 1996 at the National Institute of Biotechnology and Genetic Engineering in Faisalabad, Pakistan, and the proceedings of this meeting are summarized in Ladha et al. (1997a) and the potential research approaches for achieving improved nitrogen fixation and N use efficiency discussed at that time are summarized in Figure 108.1 (Ladha et al., 1997b).

The majority of the contributions in the latter volume deal with the isolation and characterization of associative and endophytic diazotrophs from rice and the interaction of rhizobia with rice (e.g., see Stoltzfus et al., 1997). *Nif* gene transfer to rice was also discussed (Dixon et al., 1997).

This trend continued at the Third Working Group Meeting held at the IRRI in August 1999, although at this meeting the genetic predisposition of rice for symbiotic nitrogen fixation was addressed in several chapters of the proceedings of the meeting (Laddha and Reddy, 2000), setting an important trend that continues today (see following text). In addition, participants of the meeting pointed out that rice, similar to most angiosperms, but unlike *Arabidopsis*, is capable of developing a symbiotic relationship with AM fungi and that rice has the ability to respond to mycorrhizal signals that trigger plant cortical cell division and the formation of symbiotic intracellular membrane structures. Moreover, it was pointed out that many of the legume plant early nodulin genes described at that time are present in rice (Reddy et al., 2000; Reddy et al., 1998, 1999; Kouchi et al., 1999; 2000) and selected ones induced upon mycorrhizal infection and that a subset of legume plant mutants defective in nodulation are also defective in the mycorrhizal symbiosis (Laddha and Reddy, 2000; Gough and Denarié, 2000). Thus, the rice mycorrhizal endosymbiosis was considered to be a good starting point for thinking about a nitrogen-fixing endosymbiosis in rice. Indeed, this important prediction was borne out in subsequent studies on the similarities between rhizobial and mycorrhizal symbioses with plants and the discovery of the common symbiotic signaling pathway (CSSP; SYM; see Chapters 42, 55, 110; see also following text).

In addition, at the meeting, the first demonstration of the engineering of a eukaryotic alga (e.g., *Chlamydomonas*), in which the *Klebsiella pneumoniae* NifH protein functions to complement a dark-dependent chlorophyll biosynthesis mutant, was reported (Dixon et al., 2000; see also Cheng et al., 2005).

The *nif* gene transfer to cereals such as rice was thus brought back to the forefront, and further research on *nif* gene structure and regulation was stimulated (see following text).

108.3 MORE RECENT INVESTIGATIONS (PERSPECTIVE AND PROSPECTIVE)

108.3.1 Associative and Endophytic Diazotrophs

Indigenous free-living and associative BNF systems are ubiquitous in paddy soils and offer an alternative to the fertilizer input-based technologies. Indigenous diazotrophs and soil organic N together meet more than half of the N-requirement of rice and often are sufficient for sizable

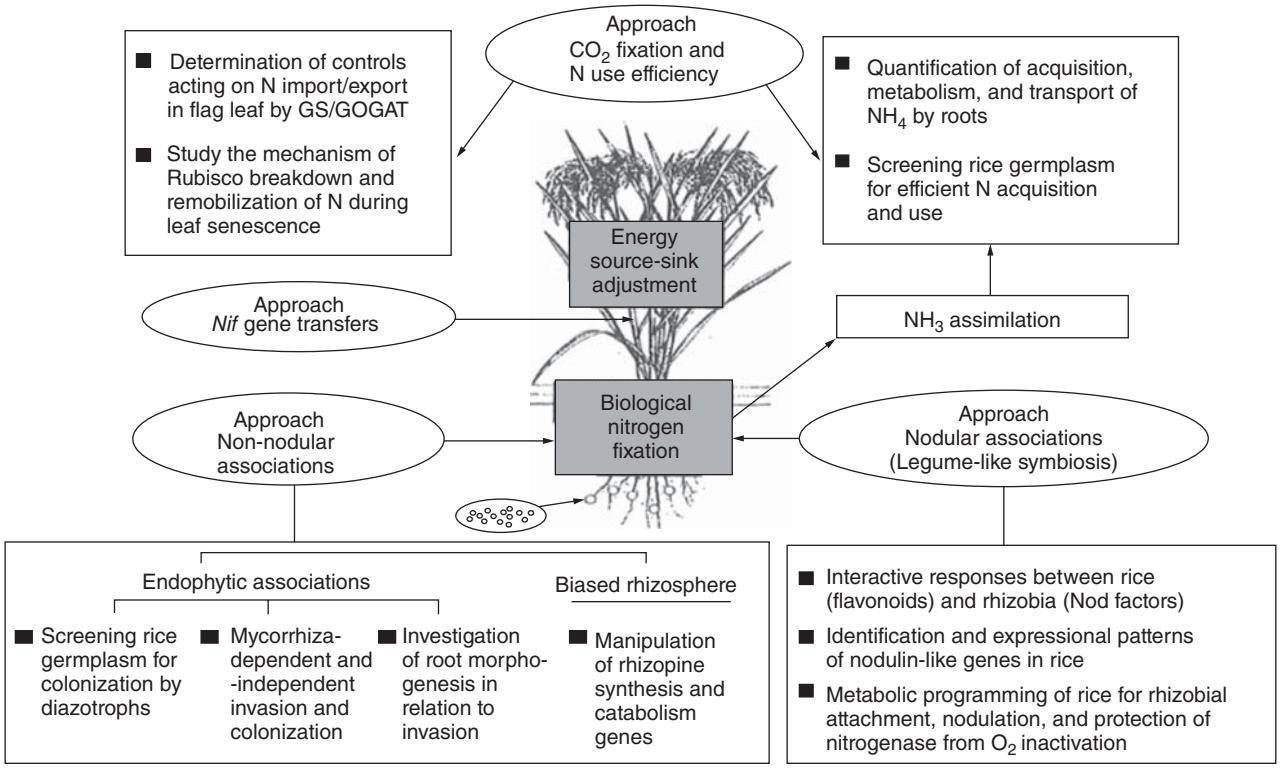

Figure 108.1 Potential research approaches for improving nitrogen fixation and N use efficiency in rice. (Reprinted with permission from Springer; Ladha et al. 1997a.)

yields, sometimes equivalent to yields obtained from using moderate amounts of fertilizer N (Ladha and Reddy 2003). The aquatic environment in rice fields provides an optimum environment for BNF by diverse free-living autotrophs and heterotrophs. The major BNF systems are cyanobacteria and photosynthetic bacteria that inhabit floodwaters and soil surfaces, as well as heterotrophic bacteria in the root zone and free-living in the soil. Multiple studies have reported the isolation and characterization of diazotrophic bacteria from rice and other cereals. However, their actual contribution of fixed nitrogen to the rice plant is difficult to measure (Ladha and Reddy, 2003), and the physiological and biochemical mechanisms that operate in these associations remain to be elucidated (Ladha, 1986). Moreover, the variability observed with different rice varieties remains unexplained. One study reported the mapping of seven genes underlying rice genotypic variability to stimulate BNF, suggesting that the ability of rice to enhance BNF in the rhizosphere is controlled by multiple genes (Wu et al., 1995; Ladha and Reddy, 2003).

In addition to associative diazotrophs, there has been an increasing interest in endogenous BNF systems, particularly nitrogen-fixing endophytic bacteria (e.g., see Stoltzfus and de Bruijn, 2000). Endophytic bacteria are defined as bacteria detected inside surface-sterilized plants or extracted from inside plants and having no visibly harmful effects on the host plant (Hallman et al., 1997; Mano and Morisaki, 2008; Reinhold-Hurek and Hurek, 2011; see also Chapter 114]. The potential of diazotrophic endophytes for BNF in plants was already recognized by the BNF Working Group (Ladha et al., 1997a; Laddha and Reddy, 2000). Various diazotrophic endophytes have been isolated from rice (for a listing, see Mano and Morisaki, 2008, and references therein). They were identified as BNF bacteria on nitrogen-free medium or as rhizobia using a legume trap. However, as in most cases thus far, an actual BNF-based growth advantage of the diazotrophic endophytes for the host rice plants has not yet been clearly demonstrated. Moreover, as is the case for many, if not most, associative or endophytic diazotrophs, growth promotion of the plant may not be due to BNF but to other factors, such as the production of hormones (e.g. *Azospirillum*; Chapters 90, 91). Thus, they should be labeled as plant-growth-promoting (PGP) bacteria (Lugtenberg et al., 2013; de Bruijn, 2013). A historical perspective on PGPR (rhizobacteria) by Elmerich can be found in Chapter 88 of this book. In this review, the extensive Brazilian experience with associative and endophytic diazotrophs and PGPR on sugarcane, rice, and other grasses is highlighted. This experience is further discussed in a special issue of plant and soil (E.K. James and J.I. Baldani, Eds. 2012, Issue 356], based on the BNF with non-Legumes International Symposium of 2010 in Brazil. In the case of sugarcane and other biofuel crops, the location of the meeting in Brazil was

particularly pertinent since the highly advanced Brazilian bioethanol program, which produces over 27 billion liters of ethanol per year, is based on the cultivation of sugarcane, deriving much of its N-requirements via BNF (James and Baldani, 2012). Field-based BNF quantitative studies revealed very substantial inputs into sugarcane and elephant grass of at least 40 kg N per ha per year for sugarcane and up to 132 kg N per ha per year for elephant grass (Urquiaga et al., 2012; De Morais et al., 2012).

Several studies in this special issue on PGPR have focussed on *Azospirillum* which has long been known to be an effective (diazotrophic) PGPR on rice and other cereals. Papers by Castro-Guerrero et al. (2012), Walker et al. (2012), and Vargas et al. (2012) have examined in detail at the biochemical and/or molecular level the mechanisms by which cereals such as rice respond to inoculation by *Azospirillum* strains and how the bacteria respond to their host. Similarly, Brusamarello-Santos et al. (2012) have examined the molecular response of rice to inoculation with another endophytic diazotroph, *Herbaspirillum seropedicae* (James and Baldani, 2012). Moreover, in Chapter 90 in this book, Yaacov Okon describes the agronomic application of *Azospirillum* inocula on maize and rice and reports the positive PGP results of extensive field experiments. The effects of the inoculation of *Burkholderia vietnamiensis* and related diazotrophic bacteria on grain yield of rice are described by Govindarajan et al. (2008), and the beneficial properties of plant-associated *Burkholderia* species are discussed by Hirsch et al. (Chapter 89 and in Chapter 17).

One of the most promising of the endophytic diazotrophic bacterial genera both as a "Model" and a prospective inoculant is *Herbaspirillum* (James and Baldani, 2012). Chubatsu et al. (2012) and Monteiro et al. (2012) have explored how *H. seropedicae* regulates N fixation and the (generally beneficial interactions between the members of this interesting and diverse genus and their various Poaceous hosts (James and Baldani, 2012). The reader is referred to these articles for further details. Infection and colonization of seedlings of two rice varieties by *H. seropedicae* Z67 were also investigated by James et al. (2002). These authors reported that CV IR42 showed a significant (approximately 30%) increase in N content above that of the inoculated controls, and it also incorporated a significant amount of 15N2 (James et al., 2002). *Herbaspirillum* attachment to maize roots is discussed in Chapter 93 of this book. The genetic and functional characterization of another PGP bacterium, *Paenibacillus riograndensis*, isolated from wheat plants, is presented in Chapter 92.

Another model endophyte which stably infects grasses such as kallar grass and rice is *Azoarcus* sp. strain BH72, studied by Reinhold-Hurek and colleagues (Krause et al., 2007). *Azoarcus* is of agrobiotechnological interest because it supplies biologically fixed nitrogen to its host and colonizes plants in remarkably high numbers without eliciting disease symptoms (Krause et al., 2007). Its ability to fix nitrogen contributes to plant growth of its original host kallar grass, and it stably colonizes rice, as well as expresses its *nifH* gene and produces nitrogenase *in situ* (Egener et al., 1999), although its BNF-mediated growth stimulation of rice plants remains to be shown.

A highly researched natural endophytic *Rhizobium*–rice association has been described by Yanni, Dazzo, and colleagues. An extensive treatise on its occurrence and ecophysiology, as well as a translational assessment of its biofertilizer performance within the Egypt Nile Delta, is presented in Chapter 111. Their studies indicate that the promotion of this association by inoculation using certain endophytic rhizobial strains can significantly enhance vegetative growth and grain yield of rice over what can be contributed by full application of N fertilizers and other plant nutrients.

The molecular biology and ecology of associative and endophytic diazotrophs have been advanced considerably by genomic and metagenomic (see de Bruijn, 2011a,b) studies. The genomes of some of the most prominent members *Azoarcus*, *Azospirillum* spp., *Burkholderia* spp., *Gluconacetobacter diazotrophicus*, *H. seropedicae*, *P. riograndensis*, and others have been determined. Moreover, the functional characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis have been reported (Sessitsch et al., 2012). These studies will reveal more details of the molecular basis of plant–microbe interactions and the observed PGP effects of the diazotrophs and other PGPRs under study.

108.3.2 Transfer of *nif* Genes to Cereals Such as Rice

The transfer of the nitrogen fixation *nif* genes to nonlegumes has been a holy grail for decades and has been discussed as a way to obtain nitrogen-fixing rice plants in the IRRI Frontier Project Working Group Meetings since their inception in 1992 and before those (Ladha et al., 1997a; Laddha and Reddy, 2000; Chapters 5 and 109). The main result of the research to date has been the seminal paper by Dixon and colleagues showing that the *K. pneumoniae* nitrogenase Fe protein gene *(nifH*) functionally substitutes for the chlorophyll biosynthetic gene *chlL* in *Chlamydomonas reinhardtii* (Dixon et al., 2000; Cheng et al., 2005). At the third meeting of the IRRI Working Group, the hope was expressed that the announcement of this advance would remove barriers to funding in this area (Ladha and Reddy, 2000]. Indeed, 12 years later, the Bill & Melinda Gates Foundation provided a large grant to advance research in the area of *nif* gene transfer to cereals, funding specifically the work by Rubio and colleagues on *A. vinelandii nif* genes summarized in the following [see also Beatty et al., Chapter 109] and the work of Oldroyd on transferring the ability to symbiotically

fix nitrogen to Maize (see following text; Oldroyd, 2013). The BBSRC (United Kingdom) and NSF (United States) have also provided funding for projects on nitrogen fixation and cereals.

More recent work has focussed on the nitrogen fixation genes of *A. vinelandii*. For a general discussion of the biochemistry of nitrogenase, see Chapter 2. The biosynthesis of the iron–molybdenum cofactor (FeMo-co) of nitrogenase has been studied in detail by Hu and Ribbe (2011, 2013) and Rubio and colleagues (Rubio and Ludden, 2008; Jimenez-Vicente et al., Chapter 7; see also references therein]. Jimenez-Vicente et al. concluded that FeMo-co synthesis is a complex process involving a number of *nif* genes that function as molecular scaffolds, metallocluster carriers, or substrate providers. The findings from their and other biochemical and genetic studies using model systems should be taken into account when designing a strategy to transfer *nif* genes into cereals such as rice. They stated that the products of at least six genes *(nifB*, *nifEN*, *nifH*, and *nifDK*) are absolutely required for FeMo-co biosynthesis and nitrogenase activity both *in vivo* and *in vitro* [Jimenez-Vicente et al., Chapter 7]. They further concluded that it is likely that the products of some genes (NifU, NifS, FdxN, NifQ, and NifV) that are required for FeMo-co biosynthesis *in vivo* could be replaced by the activities of plant counterparts. Moreover, the metallocluster carrier proteins NifX, NifY, and NafY aid in FeMo-co synthesis and insertion, but are certainly not essential and could be removed from the equation in initial approaches to carry out *nif* gene transfer [Jimenez-Vicente et al., Chapter 7]. These findings and conclusions would certainly simplify any initial attempts to transfer BNF to rice, since substantially less of the multiple canonical *nif* genes would need to be engineered into plants.

The expression pattern of the nitrogen fixation genes is another factor to be considered when contemplating *nif* gene transfer to plants. Due to the extreme oxygen sensitivity of the nitrogenase enzyme complex and energy-intensive nature of the nitrogenase-catalyzed reaction, most diazotrophs will only carry out nitrogen fixation under anoxic or microaerobic conditions and in the absence of another nitrogen source, respectively. Thus, the process is highly regulated in all diazotrophs examined in detail thus far. Examples of the regulatory circuits and proteins involved are included in this book (see Chapter 11 by Masepohl et al. on *Rhodobacter capsulatus*, Chapter 12 by Nordlund et al. on *Rhodospirillum rubrum*, Chapter 15 by Girard et al. on *Rhizobium etli*, and Chapter 10 by Lin Min on *Pseudomonas stutzeri*). Common themes and differences are discussed in these chapters. However, probably the most studied diazotroph with respect to structure and regulation of the nitrogen fixation genes (proteins) has been *K. pneumoniae* (Dixon and Kahn, 2004; Wang et al., 2013; Huergo et al., 2013). A cluster of 21 genes, organized in seven operons and carried by a sequenced 24,206 bp DNA fragment (Arnold et al., 1988) and regulated by a cascade mechanism, consisting of the NtrBC regulatory system, which responds to the nitrogen availability and modulates the expression of the *nifLA* operon. The NifLA products, in turn, control the expression of the other *nif* genes. NifL controls the activity of NifA in response to both nitrogen and oxygen status, and NifA in concert with the integration host factor (IHF) and the sigma54 holoenzyme form of RNA polymerase initiates the transcription at the other *nif* promoters (Dixon and Kahn, 2004; Wang et al., 2013]. In addition, the PII signal transduction protein plays a role in nitrogen regulation (Huergo et al., 2013).

In any case, in order to transfer the essential *nif* genes to plants, it may be necessary to remove all regulatory circuits and promoter elements, as well as intragenic or intraoperon regulatory sequences. This has been approached by two groups (Temme et al., 2012; Wang et al., 2013] using synthetic biology. In this process carried out by Temme et al. (2012), all native noncoding DNA, regulatory proteins, and nonessential genes were removed. The genes were synthesized using a new codon usage to create a divergent sequence from the wild-type genes. Recoded genes were organized into operons and placed under the control of synthetic promoters, ribosome binding sites, and terminators. In addition, a controller consisting of genetic sensors and regulatory circuits regulated the conditions and dynamics of expression of the refactored genes (operons) (Temme et al., 2012). The latter authors used the nitrogen fixation cluster from *Klebsiella oxytoca*, a *Klebsiella* isolate virtually identical to *K. pneumoniae*, and showed that the refactored *nif* gene cluster recovered 7.4% ± 2.4% nitrogenase activity of the wild-type strain, when introduced into a strain in which the full cluster was deleted.

A similar approach was used by Wang et al. (2013a) to use synthetic biology to distinguish and overcome regulatory barriers related to nitrogen fixation employing the *nif* gene cluster from *K. pneumoniae*. They stated that the expression patterns of the nif operons reconstructed were critical to the maximum activity of the nitrogen-fixing system in *Escherichia coli* and that by mimicking these expression levels with variable strength T7-dependent promoters, approximately 42% of the nitrogenase activity of the wild-type *nif* system could be detected in *E. coli* (Wang et al., 2013a). Moreover, the T7-dependent *nif* system bypassed the involvement of native regulatory factors and gave rise to substantial nitrogenase activity when cultures were grown in the presence of ammonium or glutamine (Wang et al., 2013a). The presence of oxygen was not found to inhibit *nifH* gene expression, although, as expected, nitrogenase activity was not detected in the presence of oxygen. They concluded that their manufactured *nif* cluster may provide the first step for further research into the introduction of *nif* genes into eukaryotic organelles, such as those of plants,

which has considerable potentials in agrobiotechnology (Wang et al., 2013a).

The *nif* gene cluster is not always as complex as in *K. pneumoniae*. Recently, a minimal nitrogen fixation gene cluster from *Paenibacillus* sp. WLY78 containing only 9 *nif* genes (*nifB*, *nifH*, *nifD*, *nifK*, *nifE*, *nifN*, *nifX*, *hesA,* and *nifV*) has been identified and shown to enable expression of active nitrogenase in *E. coli* (Wang et al., 2013b). This would greatly facilitate the engineering of nitrogen fixation in non-nitrogen-fixing organisms such as plants.

The other diazotroph studied extensively for its regulation of *nif* gene expression is *A. vinelandii* (see preceding text). One of the main reasons for its emergence as a model system for the biochemistry and genetics of diazotrophy is its ability to grow on N2 aerobically, its genetic tractability, and the great quantity of and quality of nitrogenase enzymes produced (Dos Santos, 2011; Dingler et al., 1988; Poza-Carrion et al. Chapter 9]. Moreover, its entire genome has been sequenced (Setubal et al., 2009; see also Chapter 22), and extensive transcriptional profiling of nitrogen fixation has been performed (Hamilton et al., 2011a, 2011b). *A. vinelandii nif* gene regulation has been studied in detail also by Rubio and colleagues (see Poza-Carrion et al., Chapter 9]. The regulatory circuits in *A. vinelandii* resemble those in *K. pneumoniae* to a large extent (see preceding text). The specific regulatory elements NifA and NifL, in concert with nitrogen control proteins GlnD and GlnK, constitute a system that integrates redox, energy, and nitrogen signals to modulate the initiation of *nif* gene expression by a sigma54 containing RNA polymerase (for details, see Poza-Carrion et al. Chapter 9 and references therein). The authors concluded that when considering the transfer of *nif* genes to a new organism, such as a plant, a decision must be made to either completely imitate the regulatory circuitry of a model diazotroph, such as *A. vinelandii*, or to abolish all intrinsic regulatory sequences and use the synthetic "refactoring" biology approach described earlier (Temme et al., 2012; Wang et al., 2013).

108.3.3 Transfer of the Ability to "NODULATE" and Fix Nitrogen Symbiotically to Cereals, Such as Rice: The "CSSP" (SYM) Pathway

It has been a long-standing goal in the field of nitrogen fixation to extend nitrogen-fixing symbioses to presently nonnodulated cereal plants such as rice (e.g., de Bruijn et al., 1995; Markmann and Parniske, 2009). Eighty percent of the stable, biologically fixed nitrogen is a direct result of the symbiotic interaction of members of the Rhizobiaceae and selected actinomycetes with leguminous, as well as nonleguminous plants. The potential of symbiotic nitrogen fixation with rice was also discussed by the IRRI Working Group in 1997 and 2000 (Ladha et al., 1997a; Laddha and Reddy, 2000). As pointed out earlier, at the latter meeting, the rice AM symbiosis was reported to share genetic elements in common with the legume–*Rhizobium* symbiosis, and therefore, the former was considered be a good starting point for thinking about a nitrogen-fixing endosymbiosis in rice (Ladha and Reddy, 2000]. This issue will be discussed in the following section.

108.3.3.1 The AM Symbiosis and the Common SYM Pathway.

The AM symbiosis is formed by plants and fungi from the Glomeromycota. The AM symbiosis dates back to about 450 million years ago and is formed by about 80% of land plants, including cereals such as rice (Harrison, 2012; Parniske, 2008; Gutjahr et al., 2008). As a result of its widespread distribution, the AM symbiosis has a global impact on plant phosphorus nutrition and on the carbon cycle. The AM symbiosis is an endosymbiosis, and during AM symbiosis, the fungus enters the root through epidermal cells and grows into the cortex where it establishes highly branched hyphae called arbuscules within the cortical cells (Harrison, 2012). At all stages of development, the hyphae growing through or differentiating within the plant cells are always surrounded by a plant membrane. This is referred to as the perifungal membrane around intracellular hyphae or the periarbuscular membrane around the arbuscule (Harrison, 2012; see also Parniske, 2008). The arbuscule is the site of phosphate delivery to the plant. Diffusible signals initiate the symbiosis before physical contact between the symbiotic partners. Strigolactones activate AM fungal metabolism and this results in hyphal branching (Harrison, 2012; Akiyama et al., 2005). This is similar to the symbiotic nodulation process where flavonoids secreted by the plant activate the nodulation genes (see following text; Chapter 50]. AM fungi secrete a mixture of sulfated and nonsulfated lipooligosaccharides (Myc-LCOs; Maillet et al., 2011) with structures similar to the product of the nodulation genes in rhizobia (lipochitooligosaccharide (LCOs) or Nod factors; Denarie et al., 1996; see also Chapters 51 and 107). In addition, active COs are produced (Genre et al., 2013), which trigger nuclear Ca2+ spiking in *Medicago truncatula* and whose production is enhanced by strigolactone. Both Myc-LCOs and rhizobial Nod factors activate genes involved in the Common Symbiotic Signalling Pathway (CSSP or SYM), leading to AM infection/colonization and nodulation (see Oldroyd, 2013; Venkateshwaran et al., 2013; see also Chapters 54, 55, 59, 110 and in the following text, Figure 108.2).

Myc factors are thought to be perceived by LysM receptor kinases in plants (Maillet et al., 2011; Venkateshwaran et al., 2013; see Chapter 51). According to this model, the binding of Myc factors to these currently unknown receptors triggers rapid responses in the plant, including calcium spiking (Chabaud et al., 2011; 2013; Oldroyd, 2013; see Chapter 54]. The perception of Myc-LCOs by these yet to be identified receptor(s) sets off the AM signaling pathway

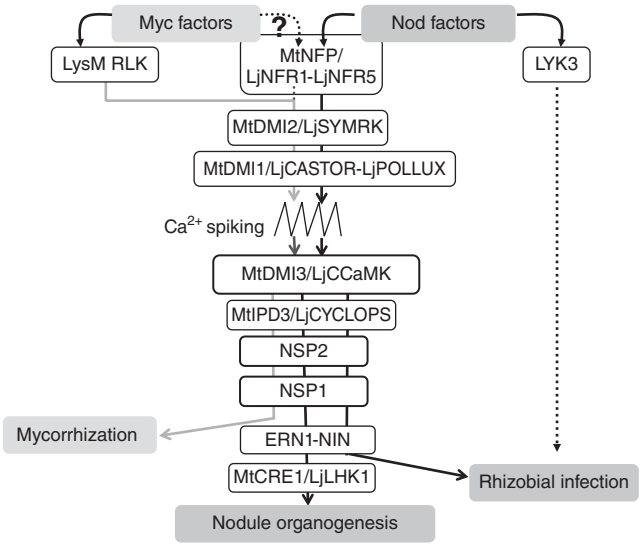

Figure 108.2 Scheme of the genetic control of AM and RL symbiotic pathways. (Reprinted from Chapter 110 by Charles Rosenberg.)

which includes an LRR receptor kinase, nuclear cation channels, nucleoporins, a calcium pump, a calcium-dependent and calmodulin-dependent protein kinase, its interacting protein, and two GRAS-family transcription factors (Venkateshwaran et al., 2013, and references therein; see also Figure 108.2). Mutant plants defective in these genes are defective for the AM symbiosis, as well as symbiotic legume-*Rhizobium* and in some cases Actinorhiza-*Frankia* interactions (see Chapters 55, 59).

108.3.3.2 The Legume–Rhizobium Symbiosis and the SYM Pathway. In addition to establishing an AM symbiosis, legumes establish an efficient symbiosis with nitrogen-fixing bacteria (rhizobia) resulting in the formation of root or stem nodules inside of which rhizobia fix nitrogen in exchange for a plant-derived carbon source (see Sections 8–10 of this book). The *Rhizobium*–legume symbiosis appeared about 60 million years ago (Ferguson et al., 2010). The induction of nitrogen-fixing root and stem nodules on leguminous plants by soil bacteria belonging to the Rhizobiaceae involves a fine-tuned interaction between the two symbiotic partners, including multiple regulatory signals produced by the bacterium and the plant to coordinate the expression of gene sets in both partners. The first step in the plant–microbe signaling pathway leading up to nodulation is the production/secretion of flavonoids, chalcones, and conjugated isoflavonoids by the plant host (see Chapter 50). These host-specific compounds are responsible for activating the nodulation (*nod, noe*) genes of the microbial symbiont. Once the rhizobial *nod* genes are activated, their gene products are involved in the synthesis of a class of compounds (Nod factors), which serve as the first return signal from the bacteria to their host plant. This class of compounds has been shown to consist of LCOs of varying lengths, carrying different side groups and substituents that play major roles in conferring host specificity (Denarie et al., 1996). Nod factors are highly similar in structure to the Myc factors discussed earlier. Nod factors, like Myc factors, have been postulated to be perceived by LysM-type receptor kinases in the host plant (Madsen et al., 2003; Radutoiu et al., 2003; 2007; Ben Amor et al., 2003; Rival et al., 2012; Smit et al., 2007; Limpens et al., 2003; Venkateshwaran et al., 2013; see Chapter 51). In the model legume *M. truncatula* the NFP/LYK3 and in *Lotus japonicus* the NFR5/NFR1 are required for nodulation and have been proposed to function as receptor pairs (see Figure 108.2; Venkateshwaran et al., 2013). Recently, in *L. japonicus* (Lj), NFR5 and NFR1 have been shown to bind Nod factor (Broghammer et al., 2012). However, in *M. truncatula* (Mt), the situation seems to be different and the LysM-RLK LYR3 has been identified, a high-affinity Nod factor and MYC-LCO binding protein (Fliegmann et al., 2013; see also Chapter 51]. The Myc factor receptor remains elusive to date probably because of genetic redundancy in the LysM-RLK family. The perception of Nod factors in legume roots induces a series of responses, including ion flux changes and membrane depolarization, rhythmic calcium spiking, cytoskeletal modification and root hair curling, and activation of cortical cell divisions (Oldroyd, 2013). A signal transduction pathway starting with the MtDMI2/LjSYMRK gene is set in motion (see Figure 108.2; Chapters 5, 110). SymRK has been shown to define a common genetic basis for plant root endosymbioses with AM fungi, rhizobia, and *Frankia* bacteria. A *Casuarina glauca CgSymRK* gene was isolated and its role in root endosymbiosis was analyzed. The results revealed that SymRK was required for both AM and actinorhiza formation in *C. glauca*, indicating shared genetic mechanisms between fungal and bacterial root endosymbioses in *C. glauca* and legumes (Gherbi et al., 2008; see also Chapter 42, 43, and 55]. It is interesting to note that in the nonlegume *Parasponia*, which acquired the ability to establish a symbiotic relationship with *Rhizobium* relatively recently, there exists a LysM-RLK-like gene, *PaNFP*, controlling the formation of a symbiotic interface with rhizobia as well as mycorrhizal fungi (Op den Camp et al., 2011; see Chapter 4]. These are all indications of a common symbiotic pathway (SYM; CSSP; Venkateshwaran et al., 2013; Oldroyd, 2013; see preceding text; Figures 108.2 and 108.3). A transcriptional analysis confirmed a conserved genetic program among AM, actinorhizal, and legume–rhizobial symbioses (Tromas et al., 2012; see Chapter 42]. A comparison with genes induced by AM fungi in *M. truncatula* and rice (*Oryza sativa*) revealed a common set of genes. A comparison with genes induced in nitrogen-fixing nodules in *M. truncatula* and *C. glauca* also made it possible to define a common set of genes induced in these symbioses. The existence of this core set is in accordance with the proposed

adaptation of ancient AMF genes for new functions related to nodulation in legumes and actinorhizal plants (Tromas et al., 2012; see Chapters 42, 55, 110).

After SYMRK, the (common) signaling pathway in legumes proceeds through MtDMI1/LjCASTOR–LJPOLLUX to calcium spiking transduced by MtDMI3/LjCCaMK (Capoen et al., 2011; Oldroyd, 2013; see Chapter 54; Figure 108.2). It is interesting to note that the specific removal of the autoinhibition domain of CCaMK leads to the autoactivation of the nodulation signaling pathway in *M. truncatula*, resulting in the induction of nodules and nodulation genes in the absence of bacterial elicitation (Gleason et al., 2006). Thus, the release of autoinhibition from CCaMK after calmodulin binding is a central switch that is sufficient for the activation of nodule morphogenesis. This, in turn, has implications for the possibility of transferring this process to nonlegumes (Gleason et al., 2006).

Subsequent steps via MtIPD3/LjCYCLOPS, NSP2, and NSP1 lead to a branch point in the Sym pathway between AM fungi and rhizobial infection and nodule organogenesis (see Figure 108.2; see Chapter 59). Details of the generation of the pathway and genes downstream of NSP1 can be found in Venkateshwaran et al. (2013) and Oldroyd (2013), as well as Chapter 59, and will not be reviewed here. The most relevant observations for the purpose of this review are shown in Figure 108.3 (Venkateshwaran et al., 2013), where the common SYM pathways between legumes and monocots such as rice are compared. Clearly, monocots (rice) capable of engaging in a symbiotic interaction with AM fungi possess equivalents of many of the common SYM pathway genes found in legumes. The functionality of the rice orthologous of the SYM pathway has been tested in various ways. One way has consisted of cross-species complementation experiments, for example, testing whether full functional root nodule functioning in a SYM pathway mutant can be restored by introducing the corresponding orthologue from a nonlegume, such as rice (see Rosenberg, Chapter 110). For example, the rice (shorter) form of SymRK, *OsSYMRK*, is sufficient to restore the AM symbiosis to a *L. japonicus symRK* mutant, but not a functional *Rhizobium*–legume symbiosis (see Chapter 110). Thus, a potential specialization of legume symbiotic genes during evolution to fulfill nodulation-specific constraints may have occurred (see Chapter 110]. DMI1 in *M. truncatula* and both CASTOR and POLLUX in *L. japonicus* (see Figures 108.2 and 108.3; Venkateshwaran et al., 2013) are essential for Nod factor-induced nuclear calcium spiking, and the orthologous genes in rice play indispensable roles in rice for the AM symbiosis (Banba et al., 2008; Chen et al., 2009). The OsCASTOR gene was found to restore both the *Rhizobium*–legume and the AM symbioses in a *L. japonicus castor* mutant (Banba et al., 2008; see also Chapter 110], suggesting that this gene is functionally conserved between legumes and rice. In contrast, OsPOLLUX could not fully restore the symbiosis-defective phenotype of *L. japonicus pollux* mutants, suggesting again a functional specialization of POLLUX specific to legumes (Chen et al., 2009; see Chapter 110). For other examples of cross-complementation studies with rice SYM pathway genes, see Chapter 110 and Godfrey et al., 2006. Thus, rice, although a monocot plant species phylogenetically distant from legumes, seems to possess the counterparts of most of the known legume SYM pathway genes (see Figure 108.3). In rice as in legumes, inactivation of these genes results in the loss or alteration of the symbiotic interaction with AM fungi. In spite of these similarities, rhizobial infection cannot be fully rescued by the orthologous rice genes in some legume mutants of the SYM pathway (see Chapter 110). The fact that some of the SYM pathway genes are functionally conserved for nodule formation but not for rhizobial entry is a new illustration of the strict genetic control of rhizobial infection. In spite of this tight control of microbial infection by the host plant, the fact that most of the SYM pathway genes have a counterpart in rice suggests that engineering cereals to form nodules hosting nitrogen-fixing rhizobia might not be an unrealistic goal, provided that the key components missing in rice are identified (Chapter 110).

Gain of symbiotic gene function experiments in rice has also been carried out. Metabolic engineering of rice with the soybean isoflavone synthase IFS gene in order to achieve rhizobial nodulation gene expression was achieved by Sreevidya et al. (2006). Studies with rhizobia demonstrated that the expression of isoflavone synthase conferred transgenic rice plants with the capacity to produce flavonoids which were able to induce rhizobial *nod* gene expression, albeit to varied degrees (Sreevidya et al., 2006; see also Chapter 50]. Moreover, transgenic rice plants harboring the pea (PSL) or wild-soybean lectin-nucleotide phosphohydrolase (GS52) genes were generated and infected with *Rhizobium leguminosarum* bv. *viciae* or *Bradyrhizobium japonicum* USDA110 (Sreevidya et al., 2005). The authors concluded that the presence of lectins, such as PSL and GS52, leads to structural modifications in cell wall organization of the root hair/epidermal cells, making them prone to localized dissolution by the hydrolytic activity of compatible rhizobia to permit invasion of the root cells (Sreevidya et al., 2005). Expression of PSL and GS52 promoted rhizobial colonization of rice plants, although no evidence of infection thread formation in the rice root hairs was observed. Thus, the genetic mechanisms enabling rhizobia-triggered infection thread formation appear to be lacking in rice (Sreevidya et al., 2005).

Thus, genetic analyses in the model legumes *M. truncatula* and *L. japonicus* support the model that legumes usurped the ancestral SYM signaling pathway to carry out the nodulation process (Venkateshwaran et al., 2013). Indeed, the majority of AM SYM pathway genes are conserved in

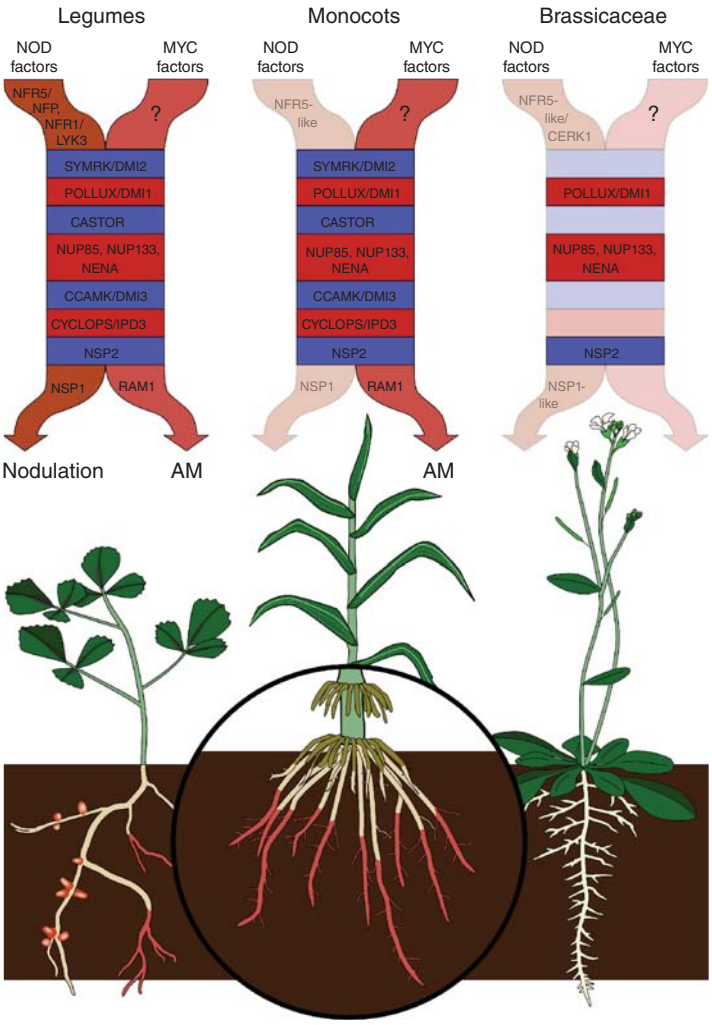

Figure 108.3 Host genetic composition decides the "social network" of plants with major root endosymbionts, such as rhizobia and AM fungi. (Reprinted with permission from Elsevier; Venkateshwaran et al., 2013.)

extant lower land plants, as well as nonleguminous plants, such as rice, suggesting an ancient origin of this signaling pathway, as well as its extraordinary degree of conservation (Venkateshwaran et al., 2013; see Figure 108.3).

Several model legume mutants mentioned earlier which are defective for AM symbiosis are also affected in nodulation, showing that these plants use the same SYM pathway for both root endosymbioses (Kistner et al., 2005; Venkateshwaran et al., 2013).

As pointed out earlier, one of the main objectives of understanding the molecular machinery of the AM fungal and rhizobial symbioses present in leguminous plants is to transfer the ability to symbiotically fix nitrogen to cereals, such as rice. One promising strategy is to use the SYM pathway machinery, which is present already in most land plants and in particular cereals, to recruit additional symbionts, such as rhizobia (Venkateshwaran et al., 2013; Oldroyd, 2013; Charpentier and Oldroyd, 2010; Geurts et al., 2012).

The choice of the *Rhizobium* (rhizobia) for these recruitment studies is under study. A number of naturally occurring nitrogen-fixing rhizobial endophytes of rice have been identified (Mano and Monsaki, 2008]. One of these diazotrophic endophytes could be chosen for rice infection studies. One could also envision choosing one of the *R. trifolii* isolates endophytic to rice described by Yanni and Dazzo (Chapter 111). In addition, a *Rhizobium* species capable of free-living and symbiotic nitrogen fixation, such as the stem- and root-nodulating *Azorhizobium caulinodans* ORS571 (Ratet et al., 1988; de Bruijn et al., 1987, 1989), could be considered. Of course, the rhizobia infecting the model legume species *M. truncatula* (*Sinorhizobium meliloti*; itself a model system for rhizobia) or *L. japonicus* (*Rhizobium loti*) are good candidates, especially since the putative Nod factor receptors (binding proteins) are being characterized (see preceding text) and the corresponding genes could be transformed into rice. Alternatively, one could choose the bacterial partners of the unusual symbiosis between photosynthetic bradyrhizobia and tropical aquatic legumes (Strains ORS278 and BTAi1; Giraud and Fleischman, 2004; Evans et al., 1990; see Chapters 28

and 45]. Besides being photosynthetic, which could provide additional energy for the symbiotic plant–microbe interaction, these strains nodulate the stem and the root of the host plant *Aeschynomene* (see Chapter 28). Moreover, they fix nitrogen in the free-living state and are found as endophytic diazotrophs associated with wild rice (*Oryza breviligulata*; Chaintreuil et al., 2000). But these strains are the most unique because they lack the canonical *nodABC* genes required for Nod factor biosynthesis (see preceding text) but maintain the ability to elicit efficient nodules on their host plant (Giraud et al., 2007; Bonaldi et al., 2011; see Chapters 28 and 45]. This revealed the existence of a Nod factor-independent nodulation process, which could be uniquely suitable to bypass a NF-dependent SYM pathway and its NF receptor in rice (Chapter 28).

In general, nonlegumes have been assumed to lack the ability to respond to rhizobial lipochitin Nod factors. However, very recent data presented by Liang et al. (2013) indicate that *Arabidopsis thaliana* plants, as well as other nonlegumes, such as tomato or corn, recognize the rhizobial Nod factor via a mechanism that results in a strong suppression of microbe-associated molecular pattern (MAMP)-triggered immunity (Liang et al., 2013). To study the interaction between symbiosis and pathogenesis, Liang et al. (2013) added both the strong MAMP flagellin 22 (flg22) and purified Nod factor from *Bradyrhizobium japonicum* to soybean leaves and showed a 25% reduction in flg22-triggered reactive oxygen species (ROS) production, the latter being a strong marker of defense response. Moreover, Nod factor or chitotetraose pretreatment decreased the activation of mitogen-activated protein kinase (MAPK), another marker for defense response downstream of flg22 recognition (David, 2013; Liang et al., 2013). Surprisingly, Nod factor pretreatment also reduced flg22-induced ROS production and MAPK activation in the leaves of the nonlegume *A. thaliana*, as well as tomato (dicot) and corn (monocot) (Liang et al., 2013; David, 2013). Similarly to legumes which recognize Nod factors through specific LysM-type receptors (see preceding text), *A. thaliana* required the LysM receptor kinase LYK3 for Nod factor recognition and plants with a mutated LYK3 gene failed to suppress flg22-mediated ROS production and MAPK activation (David, 2013; Liang et al., 2013). It is possible that Nod factor-mediated suppression of MAMP-induced immunity is universal in plants (David, 2013). Taking advantage of such potential symbiotic relationships might further the cause of establishing symbiotic nitrogen fixation in nonlegume plants, such as rice.

Otherwise, since cereals have most of the necessary components required for AM fungi infection, engineering the perception of rhizobial signals by nonlegumes is a means by which the SYM pathway could be activated to lead to preparation for infection by rhizobia (Venkateshwaran et al., 2013). Work in the only nonlegume nodulated by rhizobia, *Parasponia*, demonstrates that it is not necessary to have a complex nodule organogenesis to have symbiotic nitrogen fixation. In fact, intercellular colonization of modified lateral roots can eventually lead to intracellular fixation threads (Geurts et al., 2012; see Chapter 4]. The cross talk observed between Nod factors and the Myc factor signaling pathways already suggests that it would be possible to modify the receptor machinery to perceive rhizobial signals in cereal plants, such as rice. However, the receptors involved in Myc factor perception have not yet been identified. The transfer of the rhizobial nitrogen-fixing symbiosis to cereals mandates comparative genomic studies between plants forming nodules and those that do not. *Parasponia* is the only nonleguminous plant which associates with rhizobia and is thus available for comparative genomics with its nonnodulating close relative *Trema* [Molling and Bisseling, Chapter 4]. Such comparative studies should shed light on the genetic components which are present in nodulating *Parasponia* and missing in its nonnodulating close relative *Trema* (Venkateshwaran et al., 2013). Venkateshwaran et al. (2013) concluded that engineering important nonleguminous crops, such as rice, to accommodate nitrogen-fixing rhizobia may be an achievable goal that will have a huge impact on the sustainability of food, feed, and biofuel production. Knowledge on the ancestral and widespread CSSP or SYM pathway that has been used repeatedly in plant evolution to accommodate nitrogen-fixing bacteria suggests that this goal is quite possibly within our reach. However, ever-advancing high-throughput studies combined with in-depth genetic analyses will be critical for engineering nitrogen-fixing associations in nonleguminous crops and particularly in cereals such as rice.

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Chapter 109

Environmental and Economic Impacts of Biological Nitrogen-Fixing (BNF) Cereal Crops

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109.1 INTRODUCTION

109.1.1 The Tragedy of the Commons

In 2011, we published a review of the economic and ecological impacts of excessive nitrogen (N) fertilizer use from the global perspective, comparing regions that have reduced N fertilizer consumption with regions that have not (Good and Beatty, 2011a). N pollution resulting from excessive use of N fertilizers is an example of what the late Garret Hardin described as the "tragedy of the commons" (Hardin, 1968). Farmers, particularly in the developed world, apply high amounts of N fertilizer to maximize crop yields for economic gain. However, when this excess N fertilizer is applied to low nitrogen use efficient (NUE) cereal crops (those that take up only 30 to 50% of applied N), the remaining N is susceptible to loss by soil microbial denitrification, volatilization into the atmosphere, and leaching into waterways (Good and Beatty, 2011a; see also Chapter 108). This excess lost N fertilizer represents a direct cost to the farmers. The larger cost of using excess N fertilizer, either synthetic or organic, is represented by the damage done to aquatic and atmospheric environments. Therefore, the overall cost of excess nitrogen fertilizer use is reflected not only by the monetary cost of applying N fertilizer but also by the loss of marine biodiversity and global climate change. The severe ecological consequences of excess N use will be borne by present and future populations.

However, the knowledge that excess N fertilizer application will damage the environment is insufficient to curb fertilizer consumption for two reasons. First, producers prefer the "insurance" of having sufficient N available for maximum yield, and second, they rarely see the effects of excess N and even less often do they pay any of the associated costs. On a global scale, the incentive to continue using large amounts of N fertilizer on cereal crops is high due to our increasing human global population, livestock populations, and biofuel industry. This is compounded by the decreasing amount of arable land available for agriculture, such that maintaining or increasing crop production in available land is essential (Godfray et al., 2010; Conway, 2012).

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109.1.2 We Need Another Green Revolution, but This Time Greener

During his speech at the World Hunger Prize in 2009, Bill Gates emphasized that we need another revolution in agriculture, similar to the late Dr. Norman Borlaug's green revolution of 40 years ago, only this time greener. Such an effort must be guided by smallholder farmers and be adaptable to their localities while being sustainable to both economy and environment. Dr. Borlaug bred semidwarf wheat varieties that were resistant to rust and adapted for growth in a variety of different latitudes. These wheat varieties dramatically increased crop yields by over 150% per acre in Mexico, India, Pakistan, China, and parts of South America (Miller, 2012). Dr. Borlaug was awarded the Nobel Peace Prize for helping to avert malnutrition, hunger, and the premature death of millions of people. However, these crop varieties were selected to grow by taking advantage of the high N available when using fertilizers, and since these and other cereal crops tend to have low NUE, this means that the N that is not taken up by the plants can be lost into the environment.

There have been many calls by the United Nations, government, philanthropic funding agencies, scientists, and others for a revolution in the way we manage N fertilization of our cereal crops (EPA-SAB report, 2011; Den Herder et al., 2010; Long and Ort, 2010; ENA 2011; Sutton et al., 2012; The Montpellier Panel, 2013). We need to continue growing cereals at high yields to ensure our food security and biofuel production but in a way that is economically and environmentally sustainable. In his book "One Billion Hungry," Gordon Conway, professor of international development at Imperial College, London, optimistically wrote that we can feed the world's one billion chronically hungry people by 2050. Many of the world's hungry are smallholder farmers in developing countries (see Chapter 105). But to accomplish the goal to feed them, we will need a doubly green revolution - one that embraces both productivity and environmental sustainability. He also outlined the importance of people acknowledging that biotechnology is essential to obtain this goal. Clearly, we need to aim for an agricultural system that is stable, resilient to climate change, productive, and equitable (Conway, 2012).

109.1.3 Excess N Fertilizers and Global Climate Change

Ammonia volatilization and microbial nitrification/denitrification are N loss pathways that can have a significant impact on global warming. Urea fertilizers can be volatized to ammonia gas, especially when the urea is near or on moist, warm soil surfaces. In the nitrification pathway, bacteria oxidize ammonia fertilizer first to nitrite (NO_2) and then nitrate (NO_3), while during denitrification, other soil microbes sequentially reduces NO_3 to NO_2 and then nitric oxide (NO), which can then be oxidized to nitrous oxide (N_2O) and ultimately back to N_2 (Alberta Environment, 2010). All of these forms of nitrogen can be released in gaseous form into the atmosphere and travel large distances. N_2O, for example, is a key greenhouse gas emitted from agricultural soils and is approximately 300-fold more potent a greenhouse gas than CO_2 (Good and Beatty, 2011a). As well, N_2O has an ozone depletion potential similar to hydrochlorofluorocarbons, making this atmospheric pollutant a double threat to the environment as both a greenhouse gas and an ozone depletion catalyst (Ng et al., 2013). Loss of NH_3 from applied N fertilizers can cause soil acidification and eutrophication and when combined with NO_x in the atmosphere forms NO_3^- particulates that can affect human health and reduce visibility (Ianniello et al., 2011).

There is a need to lessen N_2O emissions and NH_3 volatilization from agricultural sources in order to mitigate global warming. Many developed countries have begun to implement plans to do this. For example, in Alberta, Canada, farmers are encouraged to lower their N_2O emissions by using the "Consistent 4R Nitrogen Stewardship" program that, if fully implemented, will generate offset credits for the farmers (Alberta Environment, 2010). This program aims to reduce N_2O emissions by optimizing the amount of N applied to the crop to lessen the opportunity for N to accumulate and persist in the soil. The program uses the 4Rs of N Stewardship (right source at the right rate, right time, and right place) and can include the use of slow-release N fertilizers, nitrification inhibitors, urease inhibitors, and variable N fertilization rates using real-time crop sensors that measure greenness and correlate this to crop N demand (please access http://www.nutrientstewardship.com/what-are-4rs for more information on the 4R N Stewardship program).

Managing N fertilizer use in cereal production has been shown to reduce N_2O emissions and NH_3 volatilization (Cui et al., 2010; Snyder and Melsinger, 2012). However, N management could be made simpler by growing cereal crops that can supply their own internal source of N fertilizer, similar to how legumes are supplied with N from diazotrophic bacteria living as bacteroids within root nodules. Cereals that have been coerced to form efficient partnerships with endophytic or symbiotic diazotrophic bacteria responsible for fixing atmospheric N_2 or that are engineered with the nitrogenase enzyme complex responsible for this catalysis could in principle supply their own fixed N, greatly reducing the need for farmers to apply external N fertilizers (see Chapter 108).

109.1.4 Why Should We Enhance and/or Introduce N Fixation in Cereal Crops?

The importance of improving both crop yield and NUE so we can meet our food needs and protect our environment

from N pollution is clear. There are many reports in the literature, from on-farm and research trials that outline N fertilizer best management practices (BMP) such as the 4Rs and variations of this depending on location (Shanahan et al., 2008; Cui et al., 2010; Alberta Environment, 2010). Cereal crop NUE has been shown to improve in research trials when BMPs have been implemented (Ladha et al., 2005). However, these improved efficiencies rarely reflect the NUE of crops on farms, which tend to remain lower (Dobermann, 2005; McIntyre et al., 2009). This discrepancy is explained as being due to lower management quality on working farms, the use of excessive N fertilizer, and more variability in the factors affecting NUE on working farms versus test plots (Cui et al., 2010). For example, Cui et al. (2010) found that N recovery efficiency was improved from 18% to 44% by using an appropriate in-season N management strategy (see also Chapter 5).

Large yield increases have been due to crop genetic modifications through plant breeding and genetic engineering. Crop improvements such as the semidwarfism, disease resistance, NUE, and tolerance to temperature, drought, and salinity have all brought about yield increases (Good and Beatty, 2011b; McAllister et al., 2012; Schroeder et al., 2013). In recent years, we have begun to see a reduction in the rate of yield increases due to plant breeding, especially in certain regions of developed countries such as the US intensive-farming corn belt where BMPs are generally followed and modern plant bred and engineered varieties are grown (Long and Ort, 2010). This suggests we may have reached the limits of what yield improvements can be bestowed by traditional breeding programs (see also Chapter 106). In order to make a breakthrough and increase crop yields further, we need to use genetic engineering to match crop N demand with crop N supply (Beatty and Good, 2011).

109.1.5 The Global Interest in Reducing N Overuse

Many government-funded technical reports have been recently written that address the increase of biologically reactive N to the N cycle from anthropogenic sources and the negative consequences this has to the environment and list methods to reduce the overuse of reactive N (EPA-SAB, 2011; ENA, 2011; Sutton et al., 2012). One of the most comprehensive is the 2011 European Nitrogen Assessment (ENA) report which was presented at the international conference "Nitrogen and Global Change" held in Edinburgh in April 2011. The report summarizes findings on the effects of excessive human-derived reactive N release into the environment on the climate, environment, biodiversity, and human health and estimates that the societal damage cost of this excess N in the EU alone ranges from €70 to 320B per year (ENA, 2011; Sutton et al., 2012).

In the United States in 2002, 65% of the human-released fixed N was from agriculture, 20% was from fossil fuels, and 15% was from industry [EPA-SAB, 2011]. This makes agriculture an obvious target for reducing the amount of human-made fixed N released into the ecosphere. The EPA Scientific Advisory Board (SAB) estimated that there could be a 25% reduction in fixed N lost to the environment over the next 10 to 20 years using existing technologies aimed at, among other targets, improving N uptake efficiency by crops and decreasing N loss pathways from agricultural lands [EPA-SAB, 2011]. We argue that further reductions in the amount of fixed N lost to the environment could be achieved by improving the synchrony between crop N supply and crop N demand (see also Chapter 5).

109.1.6 Learning from Nature

Symbiotic nitrogen (N_2) fixation by nodulating bacteria in legumes was discovered in 1886 and was closely followed by isolation of the first bacterium (*Rhizobium leguminosarum*) from a root nodule in 1888 (Franche et al., 2008; see Chapter 88). Less than 30 years later, the American scientists Burrill and Hansen asked the question, "*Is symbiosis possible between legume bacteria and non-legume plants?*" (Burrill and Hansen, 1917).

The only organisms known to fix nitrogen biologically are diazotrophic bacteria. N_2 fixation occurs through the action of an iron–sulfur–molybdenum–enzyme complex called nitrogenase. Nitrogenase catalyzes the reduction of N_2 gas (comprising approximately 78% of the atmosphere) to NH_3 (Franche et al., 2008; Lindstrom and Mousavi, 2010). The nitrogenase complex is made up of three structural proteins (NifH, D, and K). NifH (also called Fe protein) carries an iron–sulfur cluster (Fe_4S_4), and NifDK carries two iron–sulfur clusters: a P cluster and an M cluster cofactor (co) that is part of the active site and is unique to diazotrophic bacteria (Rubio and Ludden, 2008; see Chapters 2, 7). The M cluster also contains an atom of either molybdenum (FeMoco), vanadium (FeVco), or iron (FeFeco), depending on the type of nitrogenase (Eady, 1996). The nitrogenase complex is sensitive to oxygen mainly due to the Fe_4S_4 and P clusters that are irreversibly oxidized by oxygen (Bothe et al., 2010).

Aerobic–diazotrophic bacteria have developed many different ways to overcome the oxygen sensitivity of their nitrogenase complexes. For example, some Cyanobacteria (which also photosynthesize and generate O_2 as a by-product) form heterocysts where N_2 fixation occurs, which are separate from the main cells where photosynthesis takes place (see Chapter 86). Other Cyanobacteria separate photosynthesis and N_2 fixation temporally by regulating the functioning of nitrogenase so that it occurs in the dark (Bothe et al., 2010). *Azotobacter* species have the highest rate of respiratory metabolism known in bacteria. This means that

the cell is using oxygen in respiration so rapidly that there is a very low level of free oxygen within the cell. Some *Azotobacter* species also make the Shethna protein (also called FeSII) which protects the conformational shape of nitrogenase, while still others make a polysaccharide called alginate that forms a capsule around the cell and limits the diffusion rate of oxygen (Moshiri et al., 1994; Maier and Moshiri, 2000).

Depending on the species of diazotrophic bacteria, there have been up to 82 nitrogenase genes found. However, the number of genes essential for nitrogenase activity was recently shown to range from 12 to 20 in *in vitro* and *in vivo* studies (Curatti et al., 2006; Curatti et al., 2007; Curatti et al., 2008; Temme et al., 2012; see Chapter 108).

Diazotrophic bacteria have been found as free-living bacteria in low-N environments, associative bacteria that grow within plant rhizospheres, and endophytic bacteria that colonize the interior of plant roots or stems. They are also found as symbiotic bacteria that infect specific plants such as legumes, actinorhizal plants, and *Parasponia*, forming specialized bacteroids that provide fixed nitrogen to the plant host in exchange for photosynthates (Franche et al., 2008). Symbiotic biological N_2 fixation (BNF) can also provide significant amounts of the required supply of N to the plant. Symbiotic fixed N that is taken up by plants has been measured to be as high as 300 kg N/ha, which matches the synthetic N fertilizer rates of intensive wheat–maize farmers in the North China Plains (Ju et al., 2009; Cui et al., 2010).

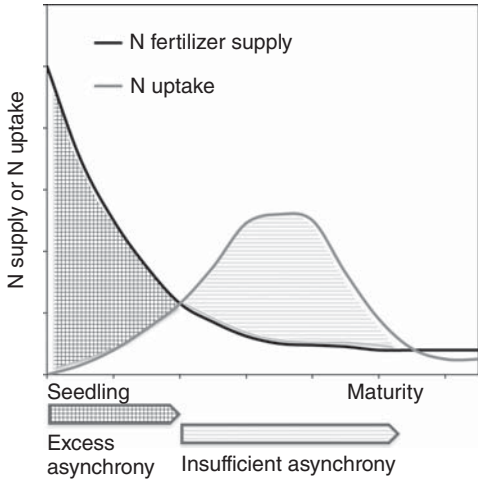

Figure 109.1 Asynchrony of N supply with N uptake. At the seedling stage, N fertilizer tends to be applied at a rate that is higher than the N demand and uptake of the seedlings, leading to an excess of N fertilizer. By midgrowth stage, the N fertilizer supply still available to the crops tends to be lower than the N uptake rate, leading to insufficient N supply.

109.2 DISCUSSION

109.2.1 Asynchrony, Excess, and Insufficient N Supply

Two reasons for low worldwide cereal NUE are, first, uniformly applying N fertilizer regardless of the actual N needs of the crop, which will often vary due to localized soil N levels, and, second, the fact that the crops' N needs increase over time (Shanahan et al, 2008). Asynchrony is the mismatch between N supply to crop N demand, leading to significant potential losses of N to the environment (Figure 109.1; see Chapter 5).

A key reason (from an N fertilizer management perspective) for the low NUE of cereal crops worldwide is poorly synchronized N fertilization management, which is often dictated by other reasons (such as when it is easiest to apply N fertilizers). In many parts of the world, the majority of N fertilizer is applied prior to planting, resulting in the largest supply of N to the plant when it is taking up a small amount (or zero) of N, representing excess N synchrony (Figure 109.1, Shanahan et al., 2008; see Chapter 5). What would be considered excess N at the preplant or sowing stage can quickly become insufficient N at a later stage of plant development. Insufficient N occurs when the crop biomass, and subsequently crop N uptake, is rapidly increasing but there is insufficient N available in the soil to meet this demand. This is because the preplant or seed stage applied N has leached down to a soil level that the crop roots cannot reach, has been lost as runoff, or has been denitrified and released back to the atmosphere as NOx, N_2O, or N_2 gases or volatized to NH_3 gas.

109.2.2 N Leaching from Various Cropping Systems and Mitigation Methods

One agronomic method to reduce N leaching further down the soil profile and into waterways, especially after a drought year, is to grow winter cereal cover crops to act as N scavengers that will take up (capture) residual soil N between growing seasons (Snyder and Melsinger, 2012). Numerous studies using rye, ryegrass, and winter wheat as cover crops show a range in the reduction of NO_3^- leaching from the field soils of 13 to 94%, with an average of 58% reduction in nitrate–N leaching. This method of conservation also has the added benefits of retaining moisture levels in the field, increasing soil carbon content, and reducing the phosphorus (P) surface runoff. However, there are limitations to this approach to mitigate N losses. For example, many farms are in geographical locations where the soil and cover crops will freeze in the winter. In addition, there needs to be enough rainfall to allow for the winter crop to mature in a timely manner so that planting of the cash crop is not delayed. In addition to these challenges, farmers would need to pay the

initial investment costs of winter cover crop growth (Snyder and Melsinger, 2012).

109.2.3 Methods to Fix and/or Avoid Asynchrony

There are methods to mitigate both excess and insufficient N asynchrony in cereal crop production such as improved agronomic management of N fertilizers, plant breeding/variety selection, and genetic engineering, as described in the following (Dobermann and Cassman, 2005; Crew and Peoples, 2005; Cui et al., 2010).

Using best nutrient management strategies, such as the 4Rs, can allow for N supply to be close to N demand. The European Union (EU) adopted the Nitrate Directive in 1987 to reduce the amount of N pollution via runoff and leaching into drinking water. In this directive, EU farmers need to show that they are using BMP by reporting detailed farm N budgets before they can receive "Common Agricultural Policy" (CAP) subsidy payments (EU Nitrates Directive, 2010).

Another method to synchronize N supply with N demand is to use symbiotic rhizobia–legumes in cropping rotations as a source of green manure (Drinkwater et al., 1998; Crews and Peoples, 2005). Legume N residues tend to mineralize N and make it slowly available for plants. This suggests that there will be some N available for the plants during all growth stages (Figure 109.2).

Adding perennial varieties that have much longer roots than cereal crops into the agricultural cropping system could reduce loss of N from the soil by leaching down the soil profile (Crews and Peoples, 2005). The long roots can uptake N from soil depths that the annual crop root systems cannot.

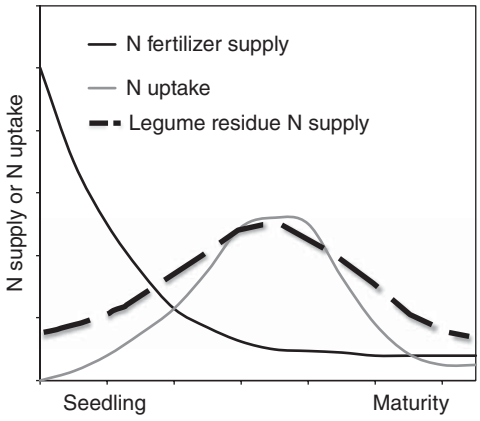

Figure 109.2 The legume residue N supply overlaid onto the N fertilizer supply and N uptake lines showing that legume residue N remains available in the soil to crop plants at a higher level than synthetic N fertilizers and can substantially reduce insufficient asynchrony between the supply and uptake of crop plants in midgrowth.

Improving the NUE of the cereal crop plants would allow for less N fertilizer application, and there are some transgenic plants that have shown NUE phenotypes (Giller et al., 2004; for a review, see McAllister et al., 2012). However, improving NUE does not address the key problem of asynchrony of N demand to N supply.

109.2.4 Development of a New System: The Introduction of BNF to Cereal Crops

The goal of BNF cereals is to synthesize biologically active N within the plant, so that N supply is tightly coupled to growth. Given the economic importance of fixed N_2 and the significant environmental damage caused by excessive N, it is time to refocus our efforts and resources on making crop plants that can fix N_2, either independently or in association with bacteria (Saikia and Jain, 2007; Cheng, 2008; see Chapter 108). If successful, these advances will start a truly "green" revolution, one that addresses the increasing demand for N in agriculture while also dramatically reducing the environmental costs associated with application of N-based fertilizers (New Scientist, 2011).

Engineering cereal crops to fix their own N could relieve the application of N fertilizers and allow the crops to synchronize their supply of N with their own demand (Figure 109.3; see Chapter 108).

While many promises of generating N_2-fixing cereal plants have been made, there has been little progress in this area from either the public or private sectors. This has

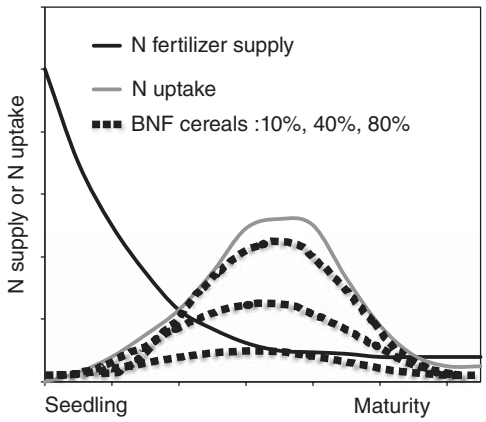

Figure 109.3 Potential for asynchrony between N uptake needs of the cereals with the supply of N available for the cereals with the development of cereal plants that can fix their own nitrogen using atmospheric unreactive N as the N source. Even a modest rate of cereal-sourced BNF would benefit the plant during the midgrowth stage where there is generally less N supply available to the plants in the soil from N fertilizers applied at the seedling stage. The economy and environment would also benefit from less leached and volatized N released into the ecosystem.

resulted in skepticism that N_2 fixation could be introduced into cereals and stalled funding in this field despite the increasing need for this trait in the major cereal crops. During 1994–2000, the International Rice Research Institute (IRRI) conducted a frontier research program on assessing opportunities for nitrogen fixation in rice and generated some valuable information on rice endophytic associations and symbiosis with rhizobia (Ladha and Reddy, 2000; see Chapter 108). However, due to the long-term nature of the experiments and the lack of long-term funding, this project was discontinued.

109.2.5 Three Approaches to Develop BNF Cereal Crops

Three different approaches to introducing BNF into cereal crops have been outlined in the literature (Table 109.1; Ladha and Reddy, 2000; Beatty and Good, 2011; see Chapter 108).

These approaches either involve the plant receiving fixed N from a diazotrophic partner in either an associative, endophytic, or endosymbiotic relationship or producing fixed N *in planta* using a stably inherited nitrogenase enzyme complex (see Chapter 108). If we engineer cereals to fix their own N, we could greatly reduce or eliminate the need for N fertilizer applications in countries with access to N fertilizers. Furthermore, we could greatly increase yields for smallholder farmers in developing countries who grow crops on poor soils with low to no N fertilizer available to them. N supply and cereal crop N demand would be synchronized. The specific challenges inherent to these approaches, the current state of knowledge in these areas, and the funding granted to these approaches are briefly discussed.

109.2.6 Enhance Endophytic Relationships with Cereals

Diazotrophic bacteria can partner with cereal crops to form surface associations or colonize the roots or stems and grow endophytically (Mahdi et al., 2010; Reinhold-Hurek and Hurek, 2011; Santi et al., 2013). These partnerships can provide some fixed N to the plant from the associated bacteria, and increases in cereal yields from diazotrophic associative and endophytic nitrogen fixation have been reported (Bhattacharya and Jha, 2012; Santi et al., 2013). Bacteria that colonize the plant rhizosphere, called rhizobacteria, have been shown to promote plant growth by various means, including N fixation, and are often described as associative plant growth-promoting rhizobacteria (PGPR)

Table 109.1 Comparison of the 3 major BNF systems evaluated and the relative benefits and challenges of each approach (see also Chapter 108)

System	BNF Potential	Amount Fixed N (kg/ha/yr)	Advantages	Disadvantages	Time	Probability of Success	Potential Returns
Endophytic diazotrophic–cereal plant enhancement	Low to medium	1–25,~ 50	Many known endophytic and diazotrophic bacteria–cereal crop partnerships in nature	Nonspecific, low population of endophytes, poor active transfer of fixed N to plants, difficult to manage, seasonal reinoculation needed	Short to medium	Medium to high	Low/medium
Endosymbiosis with diazotrophic–cereal plant partners	High	75–300	High BNF levels in nodules, symbiosis with rhizobia a well-known system, N supply synchronized to N demand	Complex genetic engineering, requires interactions between two partners, some steps in symbiosis not yet understood	Medium to long term	Low to medium	Medium/high
Stable introduction of nitrogenase in plant organelle	High	ND	Technology in the seed, broad application to crops, N supply synchronized to N demand	Complex genetic engineering into a plant organelle, challenges with oxygen levels and energy requirements	Long term	Low to medium	Medium/high

Original Sources: Ladha and Reddy, 1995, Reddy et al., 2002.

(see Chapters 88, 108). Other diazotrophic bacteria colonize the inside of the plant and live endophytically, possibly as a means to protect their nitrogenase enzyme from oxygen. N_2-fixing endophytic bacteria forming associations with some cereal crops have been found in numbers as high as 10^8 colony-forming units in roots and can provide the plants with fixed N at rates of 1–25 kg N ha^{-1} (Stoltzfus et al., 1997, Yanni and Dazzo, 2010; see Chapter 111). PGPR can also provide additional benefits by supplying other molecules such as phytohormones to their hosts. Many different PGPR are used in biofertilizer (bioinoculant) formulations that are on the market as yield enhancers (Santi et al., 2013). One approach to make BNF cereals would be to improve diazotrophic, endophytic bacteria–cereal plant associations (Beatty and Good, 2011; Santi et al., 2013). This method has the benefit that the interactions are already present in nature and these bacteria are known to promote plant growth. Farmers could include the enhanced endophytic N-fixing bacteria with the seeds at planting time.

The enhancement of these associations would need to address such factors as increasing the stability of the association by optimizing the plant and bacteria interaction and encouraging the bacterial partner to share its fixed N with the plant. There have been numerous genomic and transcriptomic studies performed on both the associative diazotrophic PGPR bacteria and cereal plants that have shown the responses to partnership from both sides (Santi et al., 2013). This information can be used to enhance these partnerships. However, the limitation of this approach to make BNF-enhanced cereals is that currently the low rate of N fixation from these associative bacteria may not be able to be increased enough to allow for a substantial reduction in N fertilizer application in intensive farming. Conversely, even modest increases in BNF capability within cereals would greatly increase crop yields in chronically N-poor regions such as Sub-Saharan Africa.

109.2.7 Symbiotic Diazotroph–Cereals

Several rhizobia and *Frankia* bacterial species have independently evolved productive nitrogen-fixing symbioses with more than 14,000 Leguminosae species, several *Parasponia* species, and approximately 200 *Actinorhizal* species (Lindstrom and Mousavi, 2010; Santi et al., 2013). These symbioses result from nitrogen-fixing bacteria colonizing the plant root cells and forming a plant nodule. Another approach to BNF cereals is to engineer cereal crop plants and/or nodulating diazotrophic bacteria to form symbiotic partnerships (Charpentier and Oldroyd, 2010; Beatty and Good, 2011; see Chapter 108). There have been significant advances in understanding how these symbioses are established, which can help us determine how to engineer this process in cereals. There are four basic steps that need to be either co-opted or introduced into cereals for cereal–diazotrophic bacteria endosymbiosis to occur. The plant and bacterial partners need to locate each other, nodulation needs to occur, the plant needs to be stably infected with the bacterial partner, and the nitrogenase complex within the bacteria needs to be protected from free oxygen. BNF researchers have recently discovered that all plants have the capacity to attract, interact, and form symbiotic partnerships with arbuscular mycorrhizal (AM) fungi (Maillet et al., 2011) via a dialog with plant-derived and fungi-derived macromolecules. It appears that rhizobia adopted or mimicked the macromolecules from AM fungi to form their own symbioses with plants at some point during their evolution (Gherbi et al., 2008; Op den Camp et al., 2011). For nodule formation, some of the genes necessary for this already exist in cereals (see Chapter 108). However, they may not be expressed in the right concentration, at the right time, or in the right place to allow for nodules to form currently (Charpentier and Oldroyd, 2010). The promoter–gene combinations for production of the correct hormones and transcription factors required for nodulation would need to be genetically engineered into the cereal.

There are endosymbiotic relationships between plants and diazotrophs that use prenodules instead of rhizobial/legume-like nodules. This type of primitive structure may be easier to introduce to cereals initially (Laplaze et al., 2000). Infection of the plant host by the endosymbiotic bacterial partner has been seen to occur in simple to very complex ways (Madsen et al., 2010). Many *Rhizobia* and *Frankia* species use a highly specialized plant-derived structure called an infection thread to colonize plant roots. A simpler infection method, called intercellular infection, is carried out by the bacterial partners of some legume species, all *Parasponia* species, and most actinorhizal plants (see Chapter 4). Mutant legumes that cannot form infection threads can still be colonized by rhizobia by the simplest method, called crack entry. All of these infection methods still allow for N fixation; therefore, the less genetically complex intercellular or crack entry methods may be more successful (Madsen et al., 2010). The nitrogenase enzyme needs to be protected from oxygen in order to function. In the rhizobial–legume symbiosis, the intracellular rhizobia develop into organelle-like bacteroids that are packed into microaerobic nodules containing leghemoglobin that sequesters free oxygen (Soupene et al., 1995). By contrast, *Frankia* fix nitrogen *in planta* in specialized vesicles that provide the oxygen protection needed for nitrogenase to function (see Chapters 35, 43). Endosymbiotic N fixation rates are often comparable to the N fertilizer rates used in intensive farming, suggesting that if cereals could be engineered to allow for diazotrophic colonization and nodulation, they could have a high level of *in planta*-sourced N to use in biomass and yield. The benefits of this BNF cereal crop approach to farmers would be that they could grow

cereal crops like legumes. N fertilizer applications to the growing crop could be greatly reduced or even eliminated provided the correct symbiotic bacterial partner was used to inoculate the cereal crop (Ng et al., 2013).

Researchers at the John Innes Centre in the United Kingdom have recently received a grant of over $9 million USD from the Bill & Melinda Gates Foundation (B&MGF) to study the feasibility of developing diazotrophic symbiosis with traditionally grown Sub-Saharan maize (www.foundationcenter.org/gpf/foodsecurity; see also Chapter 108).

109.2.8 Introduce BNF Genes Directly into Plants

Why don't plants fix atmospheric nitrogen themselves? Perhaps, it is because plants can "easily" harness bacteria to do it for them, for the price of providing photosynthate to the bacteria. Or maybe, there is a fitness-related issue inhibiting plant BNF. Whatever the reason, plants do not fix their own nitrogen, although there are thousands of different species of plants that can form endosymbiotic relationships with many different species of diazotrophic bacteria. Moreover, these plants have developed the ability to deliver C to the nodule and transport and utilize the N that is fixed in that organelle. Unfortunately, many of the crops that the world population relies on for food, such as maize, rice, wheat, and barley, do not form endosymbiotic relationships.

The third approach to engineering BNF cereals that would address many of the issues associated with excessive N applications but would be just as challenging and as lengthy a research process as engineering symbiotic cereals would be to introduce the genes encoding nitrogenase directly into plants (Merrick and Dixon, 1984; Beatty and Good, 2011; Ng et al., 2013; see Chapter 108).

The 10 to 20 bacterial genes that are essential for producing a functional N_2-fixing enzyme complex could be used to stably transform cereal crops so they could fix their own N using freely available N_2 from air (see Chapters 7, 108). There are three known types of nitrogenase that differ in the type of transition metal atom present in the active site M cluster cofactor: molybdate (Mo, with *nif* genes), vanadium (V, with *vnf* genes), or iron (Fe, with *anf* genes)(Schwarz and Mendel, 2006; Rubio and Ludden, 2008; see Chapter 2). The nitrogenase complex is made of three structural proteins (HDK) encoded by three genes *(*e.g., *nifHDK* for Mo–nitrogenase) (Dixon and Kahn, 2004; Dos Santos and Dean, 2011). Although nitrogenase is unique to bacteria, there are Fe–S containing enzymes found in the nucleus, cytoplasm, chloroplasts, and mitochondria of plant cells (see Chapter 6). For example, many plant mitochondrial enzymes function with Fe–S–P clusters. Although the M cluster cofactor (e.g., FeMoco) is unique to bacteria, plant mitochondria do make cofactors containing molybdenum (Moco). Thus, mitochondria are known to take part in the import of Fe, S, and Mo into the mitochondria, although the identity of these transporters is currently unknown. Plants are poised to be the model organism of choice to study these transporters since the single-celled eukaryotic model organism *Saccharomyces cerevisiae* does not have the Moco biosynthetic pathway (Balk and Pilon, 2011).

There are several main challenges to engineering BNF directly into cereal crops that must be overcome: first, determining which *nif* genes are essential for production of a functional nitrogenase and from which diazotrophic bacterial source; second, engineering optimal transcription, translation, and assembly of the bacterial gene-encoded metalloproteins to allow for functioning N_2 fixation within the plant; third, protecting the nitrogenase complex from free oxygen that would disable BNF function; and, finally but by no means insignificant, providing enough reducing power and energy, in the form of electrons and ATP, for the N_2 fixation reaction to occur.

Most of the genetic complexity of nitrogenase originates with the M cluster cofactor which is exclusive to nitrogenase. The sequenced, mapped, and annotated *Azotobacter vinelandii* genome has 82 nitrogen fixation (*nif*) genes located in four separate locations on the genome that encode the three types of nitrogenase (Dos Santos and Dean, 2011; Hamilton et al., 2011). This suggests a high level of genetic complexity. However, Curatti *et al*. (2007) has synthesized FeMoco *in vitro*, showing that this complex cofactor can be made using three Nif proteins plus iron, molybdate, sulfur, homocitrate, and S-adenosylmethionine (see also Chapter 7). Therefore, although the N fixation genetics appears to be made up of a large number of genes, only a small portion of these genes may need to be transferred to a plant organelle to have a functional nitrogenase enzyme complex present. Synthetic biology approaches have been used to modify the nitrogen fixation gene cluster from *Klebsiella oxytoca* (Temme et al., 2012; see Chapter 108). Certain structural features of the gene cluster were altered; all regulation, noncoding sequences, and nonessential genes were removed, and codons were modified to distinguish the modified DNA sequence from the native sequence. The genes were then placed under the control of synthetic promoters, ribosome binding sites, and terminators. This modification simplified the regulation and allowed a specific function to be assigned to each genetic component (Temme et al., 2012). This refactoring of the gene clusters from *K. oxytoca* showed that 12 *nif* genes were essential to form a functional nitrogenase in the non-N-fixing bacteria, *E. coli*, to allow it to fix N. The rapid advances in synthetic biology means we can now synthesize novel genes, regulatory components, and pathways; introduce them into the organisms of our choice; as well as target them to the appropriate cellular compartment.

Due to the oxygen sensitivity of the nitrogenase enzyme, it would need to be stably introduced into a microaerobic environment in the plant to ensure functionality, such as an organelle. Chloroplasts and mitochondria are two logical places to transfer nitrogenase genes to for many reasons; their bacterial origins may allow for a more "prokaryotic-like" transcription and translation of the genes into functional gene products, and both generate enough ATP and reducing power for nitrogenase function. As well, the chloroplast genomes of ferns, mosses, and gymnosperms encode protochlorophyllide reductase, an oxygen-sensitive enzyme that is structurally and functionally related to nitrogenase (Fujita and Bauer, 2000, Muraki *et al.*, 2010).

Although the potential rate of cereal crop BNF is unknown, the goal would be to make it possible to match the rate seen in legume symbiosis. Fundamentally, the key benefit of this approach to farmers would be that the plant itself can meet its own N demand so there would be no need to introduce an N-fixing partner at the time of sowing and N fertilizer application could be greatly reduced or eliminated (Ng et al., 2013).

109.2.9 Why Develop BNF Cereal Crops?

There has been renewed interest in bioengineering N_2-fixing cereals, and significant advances in our understanding of the biology of N_2 fixation have been made in the last two decades (Godfray et al., 2010, Oldroyd et al., 2009, Den Herder et al., 2010). The advances in different experimental model systems, including several N_2-fixing bacteria (various rhizobia and *Frankia* species), legumes (*Medicago truncatula* and *Lotus japonicus*), and crop plants (*Brachypodium distachyon*, *Zea mays*, *Oryza sativa*), are important drivers for furthering N_2 fixation in nonlegume plants. Scientific and technological advances that are critical for moving toward N_2-fixing cereals include efficient cereal transformation protocols, development of model organisms that are efficient in terms of molecular biology protocols, and genome sequencing. Access to complete genome information allows researchers to identify homologous genes across species and to identify novel genes. We also now understand nitrogenase biosynthesis from the gene to the protein level (see Chapter 2).

Why should we push ahead now when in the past we failed? We are well aware that projects of this nature are challenging and that success will come in small steps. For example, the first step in many of these concepts will be to initially demonstrate N_2 fixation in the cereal without being concerned about the amount of N_2 fixation. A small increase in available N to a plant can still have a significant effect and would be particularly valuable in areas such as sub-Saharan Africa where N fertilizer application rates are low due to cost and poor infrastructure (see Chapter 105). As well, if one could closely couple N and C metabolism, excess N would not be lost from the roots into the environment. While there is clearly a significant risk of failure of any one of these approaches, the potential benefits are enormous. Even if unsuccessful, the fundamental knowledge gained from such an attempt may prove crucial to further understanding other crop processes, such as root–pathogen interactions and nitrogen metabolism in general.

109.2.10 Is There a Yield Hit from BNF?

The idea of trying to engineer cereal crops to fix their own nitrogen is not a new one. One of the arguments against developing associative/endophytic or symbiotic BNF cereal crops is the question of whether there would be a yield penalty. In a diazotrophic bacteria–plant partnership, the plant receives fixed N but in turn supplies carbon from photosynthesis to the bacteria. Studies have shown that photosynthate C is transferred to the bacteroids in the nodules of legumes within one hour of CO_2 fixation (Voisin et al., 2003). This shows the tight connection between the two symbiotic partners for C and N production and transfer. Symbiotic nitrogen fixation specific activity is dependent on photosynthesis and nitrate availability and will correlate with both of these factors, along with oxygen levels (Voisin et al., 2003). The calculated C costs to pea plants that were directly attributed to nitrogenase activity were 1.5–4 grams C per gram of fixed N (Voisin et al., 2003). However, there have been many reports in the literature showing shoot biomass increases, seed biomass increases, and yield increases in rice, maize, and wheat grown in field and controlled conditions from inoculation with diazotrophic PGPR such as *Azoarcus*, *Azotobacter*, *Pseudomonas*, *Burkholderia*, and other bacterial species (for a review, see Santi et al., 2013). This would suggest that although a plant–diazotrophic bacterial partnership will cost the plant C, the plant gains fixed N and potentially other beneficial molecules that increases yield instead of costing yield. Second, the whole concept of N limitation is based on the fact that when N is applied to crops, they are clearly not C limited, in that they can quickly grow and increase both size and yield. Third, recent efforts underway to improve the rate of C fixation in plants may provide any needed boast in the fixed C required to allow increased growth (Parikh et al., 2006). However, the question of whether there would be an energy deficit in the BNF cereal plants remains to be studied once the nitrogenase genes are engineered into cereal plants.

109.2.11 Economic Benefits of BNF Cereal Crops

Conservation and resource management specialists discuss achieving the equity, economic, and environmental triple bottom line as the ideal outcome of conservation (Halpern et al.,

2013). This is where social outcome, equity, and conservation goals are maximized, while costs to reach those goals are minimized. Conservation studies have shown that the degree of success in environmental protection is highly correlated to the level of perceived or real equity (or compliance) of the participants (Halpern et al., 2013). When equity is low, the probability of success in the conservation project is also low. Providing equity to the participants who have a significant stake in the outcome of a conservation plan, by involving them in the decision making, greatly increases the motivation to adhere to a plan, which in turn increases the success rate. The need to reduce N fertilizer use while continuing to increase yields, in order to minimize the external costs from loss of excess fixed N into the environment, could be analyzed using triple bottom-line conservation models to help determine how best to approach this problem. The equity issues for N pollution from excess N fertilizer use involve the inequitable availability of N fertilizers to farmers and the inequitable cost of N pollution from excess N fertilizer use to the *global commons*. Some farmers cannot access or afford to buy N fertilizers (such as smallholder farmers in developing countries), while other farmers (such as intensive farmers in developed countries) have readily available access, although it is an expensive commodity. Equity, in the case of N loss and environmental damage, is complicated because N loss is difficult to quantify since it fluctuates depending on climate and soil conditions. The source of N pollution is difficult to trace as it enters fast-moving environments such as air and water such that N pollution causes environmental damage that is both spatially and temporally separate from the initial source. Farmers are the initial economic stakeholders in decision about the application of N fertilizers in terms of a simple use/not use and subsequent high/low yields. However, we all become stakeholders in the outcomes of farming, which include food security and the environmental damage caused by excess nutrients. Introducing cereal varieties to farmers on a sliding cost scale that matches their economic resources that have built-in N fertilizer reduction potential could help address the complex equity problems that threaten to derail the goal of decreasing N pollution.

We previously calculated the external costs associated with excess N fertilizer consumption to be a minimum of 44% of the direct costs associated with excess N fertilizer application (Table 109.2; Good and Beatty, 2011a).

This is based on expenses associated with global warming from N_2O emissions, soil acidification and eutrophication costs from ammonia volatilization, and maritime industry and property losses from nitrate runoff and leaching. The ENA (2011) calculated the external costs of excess N fertilizer use for the EU to be much higher, with the annual EU environmental costs ranging from €70B to 320B euro. These two costs were used to calculate the lower and upper limits of the total environmental costs associated with the N fertilizer amount determined to be excess in Table 109.2. These calculations predict the global environmental costs in 2020 to range from $8.7B USD to $251B USD if the status quo is maintained. However, if we could introduce BNF to cereal crops, we believe that with sufficient efficiency enhancements, it may be able to possibly supply from 25 kg N per hectare up to 300 kg N per hectare to the cereal crops from an *in planta* source. This could reduce N fertilizer consumption from the developed world cereal farms and increase cereal crop yields from the developing world farms. If we assume 150 kg N per hectare as an average N fertilizer application to cereals, then supplying a range of 25 to 300 Kg N per hectare as *in planta* N would let farmers reduce their N fertilizer application by 17–100%. If we optimistically suggest that BNF cereals could reduce world N fertilizer use from 2007 levels by 10% by 2020, then we would reduce our N usage by 20 MMt. This would correspond to a direct fertilizer cost savings of $19.8B USD and reduced N_2O emissions of 0.2 MMt (taking the IPCC Tier 1 N_2O–N emissions level), corresponding to a further savings of $0.91B USD in CO_{2eq}. This would cumulate in potential total environmental cost savings of $8.7B USD to $251B USD (Table 109.2). Even for a country like the United States that uses BNMPs and technology in their intensive cereal crop farming, there would be significant cost savings by growing BNF cereals. For a country such as China that uses high levels of N fertilizers in cereal crop production, the savings from reduced N use due to BNF cereals could be very large.

109.2.12 Targeted Projects Funded to Date

Nongovernment philanthropic foundations have granted significant sums of money to improve food security for the hungry and poor; many of these people are women who are also smallholder farmers. These grants have gone to a wide range of agricultural-related projects in countries such as Sub-Saharan Africa, India, and the Philippines. Philanthropic organizations have also given grants for projects based in developed countries, such as improving agricultural education in the rural United States. A few of these philanthropic foundations, such as the B&MGF and the Howard G. Buffett Foundation, have given large grants to research organizations in various countries to improve certain crop traits such as drought and salt tolerance. They have invested approximately $1.8B USD into agricultural programs aimed at improving the lives of smallholder farmers in Sub-Saharan African countries by reducing poverty and hunger (http://www.gatesfoundation.org/Media-Center/Press-Releases/2011/11) (Table 109.3).

Many of these grants are aimed at helping women, since agricultural researchers and the B&MGF have recognized women as the main smallholder farmer in these countries and as the people most likely to be responsible for children's

Table 109.2 The predicted economic benefits of BNF cereal crops from the basis of the world, the United States, and China

Year	Actual/ Predicted Consumption (MMt N)	Value* (US$B)	Proposed Reduction (from 2005)	Reduced Use (MMt)†	Value of Reduced Fertilizer (US$B)	Reduced N_2O Emissions (IPCC) (MMt)‡	Value of Reduced N_2O Emissions§ (IPCC) (US$B)	Total Environmental Cost¶ (US$B)
World								
1990	75.8	32.2						
2005	100.6	80.0						
2020	110.7	108.5	10%	20.2	19.8	0.20	0.91	8.7 – 251.1
2050	151.6	227.4	40%	91.2	136.9	0.91	4.11	60.2 – 1133.6
2100	176.3	387.9	80%	156.2	343.6	1.56	7.03	151.2 – 1941.6
United States								
1990	9.5	4.1						
2005	14.5	11.5						
2020	16.7	16.3	10%	3.65	3.58	0.04	0.16	1.6 – 45.4
2050	19.9	24.3	40%	11.2	16.80	0.11	0.50	7.4 – 139.2
2100	23.5	35.2	80%	20.6	45.32	0.21	0.93	19.9 – 256.1
China								
1990	18.6	7.1						
2005	34.8	27.6						
2020	42.4	42	10%	11.1	10.86	0.11	0.50	4.8 – 138.0
2050	54.5	66.5	40%	33.6	50.43	0.34	1.51	22.2 – 417.6
2100	69	103.5	80%	62.0	136.49	0.62	2.79	60.1 – 770.7

[a] N fertilizer prices per ton: 2007, $795; 2030, $1220; 2050, $1500; 2100, $2200

[b] (Year actual/predicted consumption – 2005 actual consumption) × (100-ratio)

[c] Reduced N use × 0.01 N2O–N Tier 1 emissions.

[d] Reduced N_2O emissions x 300 GWP x $15 per ton CO_{2eq}.

[e] Lower limit = reduced N use x 0.44 adapted from environmental costs of excess N (Good and Beatty, 2011). Upper limit = reduced N use x $12.43B USD adapted from upper limit of environmental costs from ENA (Sutton et al., 2011).

MMt = million metric tons

Adapted from Good and Beatty (2011); Ng et al. (2013)

nutrition. These grants have allowed for the implementation of programs to increase crop yields so these farmers can both feed their family and produce enough market product. A subset of these grants are aimed at increasing the yields of crops that are traditionally grown in these regions by improving the availability of N to these crops. Table 109.3 outlines six projects funded by B&MGF (totaling over $52M USD) that involve N and agriculture in Sub-Saharan Africa. These include improvement of N fertilizer, development of NUE maize, improvement of the BNF capacity of legume crops grown, introduction of BNF directly to cereals, and development of N_2-fixing symbiotic cereals. These funded projects show a commitment to improving crop yields using both existing technologies, such as plant breeding and symbiotic legume crops, and using highly experimental emerging technologies such as N_2-fixing plant biotechnologies. The B&MGF granted nearly $10M USD to researchers at the John Innes Centre in the United Kingdom to test the feasibility of developing diazotrophic symbiosis with traditionally grown Sub-Saharan maize. They also granted over $2M USD to researchers at the Technical University of Madrid to research engineering cereals that can fix their own N_2 (see Chapter 108).

Government funding of agricultural development programs in the G20 countries have generally decreased since the 1980s, falling from 17% in 1980 to 3.8% in 2006 (Sasson, 2012). This reduction in money going to agriculture, along with other factors including grain production going to biofuel stocks and animal feed, climate change, and reduction in available arable land and fresh water, has resulted in food security crises for many countries. The G8 and G20 countries have recently recognized this deficit in agriculture funding and have made pledges to increase spending in agriculture and food-related programs in order to reduce the rising number of impoverished, malnourished, and hungry people in the world. At the Millennium Summit of the United Nations in 2000, the 193 member states and approximately 23 international organizations agreed to achieve eight international millennium development goals (MDG) by 2015. The goals were to (i) eradicate extreme poverty and hunger, (ii) ensure universal primary education, (iii) promote gender equality and empower women, (iv) reduce

Table 109.3 Agricultural and global development projects funded by nongovernment foundations that involve N, crop yields, and sustainability (for information on other projects related to food security, please see www.foundationcenter.org/gpf/foodsecurity)

Year	Granting Agency	Grantee	Project Description	Amount in USD
2012	B&MGF	John Innes Centre, United Kingdom	Test the feasibility of developing cereal crops capable of fixing nitrogen as an environmentally sustainable approach for small farmers in sub-Saharan Africa to increase maize yields	$9,872,613
2011	B&MGF	Technical University of Madrid, Spain	Support the introduction of biological nitrogen fixation in cereals in Sub-Saharan Africa and South Asia	$2,927,139
2010	B&MGF	Columbia University, United States	Support the Earth Institute's International Nitrogen Conference	$50,000
2009 and before	B&MGF and Howard G. Buffet Foundation	Wageningen University, the Netherlands	N2Africa: increases legume productivity, family nutrition, soil health, cropping systems, and farm income for small farmers in Burkina Faso, Mali, Nigeria, Ghana, Kenya, Rwanda, and Malawi Extended to Liberia and Sierra Leone	$20,506,733 (over $2 million from HGBF)
2009 and before	B&MGF	Centro Internacional de Mejoramiento de Maiz y Trigo, Mexico	Develop improved varieties of maize for higher yields on the low-nitrogen soils prevalent in Africa	$18,307,621
2009 and before	B&MGF	Massachusetts Institute of Technology, United States	Improve the chemistry of nitrogen catalysts	$393,706
	B&MGF	International Center for Tropical Agriculture	Evaluate and scale up new chemical and biological commercial products for improving and sustaining crop yields	$4,825,343
Total				$52,057,812

child mortality rates, (v) improve maternal health, (vi) combat HIV/AIDS and malaria, (vii) ensure environmental sustainability, and (viii) develop global partners for development (http://www.un.org/millenniumgoals/). Goals (i) and (vii), to eradicate extreme poverty and hunger and ensure environmental sustainability, pertain closely to agriculture development such as improving NUE and introducing BNF to cereals to increase crop yields in a sustainable way. However, it could be argued that implementing research and agricultural strategies to sustainably improve crop yields in traditionally low-yielding, developing nations would also help to achieve the rest of the goals as well. Unfortunately, meeting these goals is proving to be very challenging. In 2010, 13.5% of the world population was in extreme poverty and hunger. Achieving goal 1 would require that value to be 8% or less by 2015. The G8 countries state that they want to address global hunger, and in 2008, during the G8 summit in Italy, they pledged to raise $22B USD to combat food insecurity issues. However, achieving this level of support has proven very difficult. In 2010, a global food security program was funded ($900M USD) by the United States, Canada, Spain, South Korea, and the B&MGF. Presently,

$224M USD of this has gone to five different countries for food security issues (Sasson 2012).

109.3 CONCLUSIONS

In addressing the challenges to food production that we face now and in the future, we need to consider methods that will allow for increased crop yields but that are economical and universally available and do not add N pollutants to our ecosphere. One method that would address the need for fixed N in both developed and underdeveloped regions would be to grow BNF cereal crops (see Chapter 108). Cereals that can fix their own N from atmospheric N_2 would have their N supply matched to their N demand. In the areas of the world that have little to no access to N fertilizers, this would increase yields and result in decreased malnutrition, hunger, and poverty. In areas that have access to N fertilizers, reducing N fertilizer applications would reduce environmental damage from N pollution and directly reduce farmers' costs.

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Chapter 110

Conservation of the Symbiotic Signaling Pathway between Legumes and Cereals: Did Nodulation Constraints Drive Legume Symbiotic Genes to Become Specialized during Evolution?

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110.1 INTRODUCTION

Root endosymbioses play an important role in plant nutrition. Arbuscular mycorrhizal (AM) fungi of the Glomeromycota phylum provide improved nutrient uptake to plants and protection against stress. The AM symbiosis dates back to about 450 MY and is formed by about 80% of terrestrial plant species, including cereals (Parniske, 2008). The *Rhizobium*–legume (RL) symbiosis, which supplies combined nitrogen to the plant partner, appeared about 60 MY ago and is basically restricted to the legume family (Ferguson et al., 2010). Though these two root endosymbioses differ in numerous aspects, both rhizobial and fungal microsymbionts have to enter host root tissues and reach cortical cells to establish a functional symbiotic interaction.

The establishment of both the RL and the AM endosymbioses is coordinated by signal exchanges between plants and their microbial symbionts. Rhizobia produce diffusible lipochitooligosaccharidic (LCO) signals called Nod factors (Dénarié et al., 1996; see Chapter 51). Perception and transduction of these symbiotic signals through a specific signaling pathway play a central role in nodule organogenesis and bacterial infection, the two prerequisites for functional nitrogen-fixing symbiotic interaction (Gough and Cullimoren, 2011; Oldroyd, 2013; see Chapter 59). Similarly, AM fungi produce diffusible signals, including Myc-LCOs (Maillet et al., 2011) and also short-chain chitooligosaccharides (COs) (Genre et al., 2013) that are perceived by host plants (see also Chapter 108).

Genetic studies on the model legumes *Medicago truncatula* and *Lotus japonicus* have shown that after an initial perception step, the subsequent transduction of the symbiotic signals is controlled by a set of genes that are necessary for the establishment of both RL and AM symbioses (Venkateshwaran, et al. 2013), thus defining a common symbiotic signaling pathway (CSSP; also known as CSP or SYM), illustrated in Figure 110.1 (see also Chapters 42, 43, 54, 55). These observations support the hypothesis of the recruitment by the more recent RL symbiosis of elements of the preexisting AM symbiotic signaling pathway (Geurts et al., 2012;

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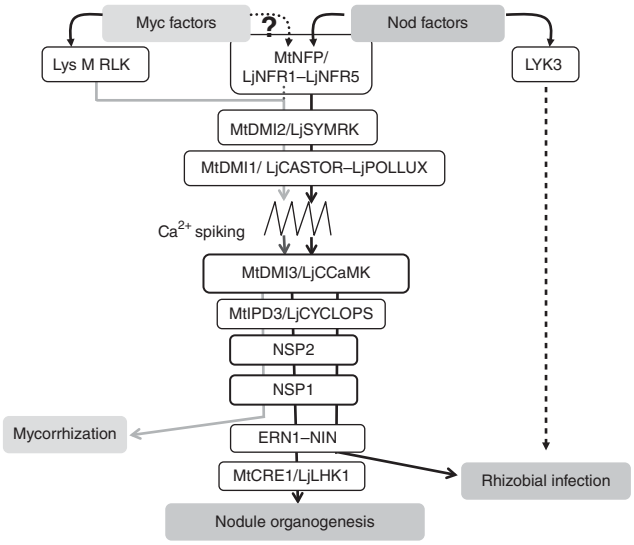

Figure 110.1 Scheme of the genetic control of AM and RL symbiotic pathways.

Markmann et al., 2008; Parniske, 2008, Venkateshwaran et al., 2012). One question arising from this hypothesis is whether functional specialization of the CSSP genes occurred during evolution of the RL symbiosis from the AM symbiosis.

To address this question, one approach has consisted in cross-species complementation experiments, that is, testing whether full functional RL symbiosis can be restored in a CSSP legume mutant by introducing the corresponding gene from a nonlegume species. Because they are nonnodulating and phylogenetically distant from legume plants, but able to establish the AM symbiosis, cereals are a convenient source for nonlegume CSSP genes, and rice (*Oryza sativa*), one of the most extensively studied cereal species, has generally been chosen for these studies.

The scope of this chapter is to summarize and discuss the results obtained by this approach to evaluate the level of specialization that could result from constraints associated with establishment of the RL symbiosis.

110.2 PERCEPTION OF THE SYMBIOTIC SIGNALS

The initial step of perception of rhizobial symbiotic signals by legume host plants, though not yet completely understood, involves lysin motif receptor-like kinases (LysM-RLKs; see Chapter 51). In *L. japonicus*, LjNFR1 and LjNFR5 are able to bind Nod factors and form a receptor complex localized in the plasma membrane (Broghammer et al., 2012; Madsen et al., 2011; Radutoiu et al., 2007). In *M. truncatula*, MtNFP, the LjNFR5 orthologue, though never shown to be able to bind NFs, is absolutely required for Nod factor perception, whereas MtLYK3, the LjNFR1 orthologue, is specifically required for rhizobial infection (Catoira et al., 2001; Gough and Cullimore, 2011; see Chapters 51, 59). Though Nod and Myc factors are structurally very similar, legumes carrying mutations in *NFP/NFR5* or *LYK3/NFR1* are still able to establish a functional AM symbiotic interaction, suggesting that they are not required for the perception of AM symbiotic signals. However, Maillet and coworkers reported that the root branching stimulation observed in response to the addition of purified Myc-LCOs was largely affected in *M. truncatula nfp* mutants (Maillet et al., 2011). Also, transcriptomic responses induced by Myc-LCOs are largely controlled by *NFP* (Czaja et al., 2012). Thus, NFP, though not essential for the AM symbiosis, could play a role in AM symbiotic signal perception. The main receptor(s) of these AM symbiotic signals could be other LysM-RLK(s), since this protein family is known to be involved both in LCO and CO perception (Gough and Cullimore, 2011). This hypothesis is further supported by studies on *Parasponia*, a nonlegume genus that acquired the ability to establish a symbiotic interaction with rhizobium relatively recently (see Chapter 4). In this plant, genetic analysis revealed that the same LysM-RLK gene, *PaNFP*, has a dual symbiotic function, controlling the formation of the symbiotic interface with rhizobium as well as mycorrhizal fungi (Op den Camp et al., 2010). Thus, the rhizobial Nod factor perception mechanism is likely to have been recruited from that used by plants to perceive AM fungi, but could have rapidly evolved into forms specific to legumes, because of particular constraints for nodulation. Because of this early specialization of RLS specific receptors in legumes, it is not expected that LysM-RLK genes from nonnodulating plant species could restore nodulation in *M. truncatula nfp/lyk3* or *L. japonicus nfr1/nfr5* mutants. However, it could be of interest to test whether the *PaNFP* gene, though not yet specialized only for nodulation, could support nodulation in legume mutants.

110.3 GENERATION OF CALCIUM SPIKING

One of the earliest cellular responses of legumes following perception of microbial symbiotic signals is the triggering of nuclear and perinuclear periodic calcium oscillations. This so-called "calcium spiking" response (see Chapters 54, 57) is induced by both Nod and Myc signals and relies on a particular subset of the CSSP genes, namely, *DMI1* and *DMI2* (DOES NOT MAKE INFECTIONS) in *M. truncatula* (Gough and Cullimore, 2011; Wais et al., 2000).

M. truncatula DMI2 (*SYMRK* in *Lotus*) is a leucine-rich repeat (LRR) receptor-like kinase (Endre et al., 2002; Stracke et al., 2002). This gene is not only essential for the establishment of the AM and RL symbioses in legumes but is also required for establishment of actinorhiza, a

nitrogen-fixing symbiotic association formed by members of the Fagales, Rosales, and Cucurbitales with Gram-positive bacteria of the genus *Frankia*. Actinorhizal plants, such as *Casuarina* or *Datisca,* carrying mutations in the *SYMRK* gene are affected for the establishment of both AM and actinorhizal symbioses (Gherbi et al., 2008; Markmann et al., 2008; see Chapters 42, 43, 55), suggesting that the actinorhizal symbiosis also evolved by recruiting genes from the preexisting AM signaling pathway. Markmann and coworkers observed that the extracellular domain of SYMRK from various land plant lineages exists in at least three different structural versions, differing by their exon composition. The "full-length" version is present in the eurosid clade, which includes all nodulating lineages, while shorter versions are found outside this clade (Markmann et al., 2008). Interestingly, in contrast to the full-length versions containing three LRR motifs, which fully restore the symbiotic properties of a *L. japonicus symRK* mutant, the rice or tomato shorter forms are sufficient to restore the AM symbiosis but fail to restore a functional RL symbiosis. When inoculated with their rhizobial symbiont *Mesorhizobium loti*, the *L. japonicus* mutant roots transformed with *OsSYMRK* exhibit primordial swellings generally devoid of bacteria. Thus, only the "full-length" version of SYMRK, the presence of which is restricted to the nodulating clade, seems to satisfy molecular constraints for bacterial penetration through infection threads (Markmann et al., 2008). The DMI2/SYMRK receptor kinases containing only one or two LRR motifs cannot support this functional specialization.

L. japonicus CASTOR and *POLLUX* and *M. truncatula DMI1* are predicted to encode nuclear potassium channels (Ane et al., 2004; Imaizumi-Anraku et al., 2005). *M. truncatula dmi1* mutants and *L. japonicus* mutants affected either for CASTOR or POLLUX are blocked at an early stage of both the RL and the AM symbioses (Catoira et al., 2000), and do not show calcium spiking in response to Nod factors (Oldroyd, 2013; Wais et al., 2000; see Chapter 54). Thus, DMI1 in *M. truncatula* and both CASTOR and POLLUX in *L. japonicus* are indispensable for NF-induced nuclear calcium spiking. The orthologous genes in rice, *OsCASTOR* and *OsPOLLUX*, have been shown to play indispensable roles in rice for the AM symbiosis (Banba et al., 2008; Chen et al., 2009). Cross-species complementation experiments showed that *OsCASTOR* restores both the RL and the AM symbioses in *L. japonicus castor* mutants (Banba et al., 2008), indicating that this gene is functionally conserved between legumes and cereals. In contrast, *OsPOLLUX* could not fully restore the symbiosis-defective phenotypes of *L. japonicus pollux* mutants, suggesting a functional specialization of *POLLUX* specific to the legume family (Chen et al., 2009). Furthermore, Venkateshwaran and coworkers showed that a *dmi1* mutant of *M. truncatula* requires both *CASTOR* and *POLLUX* from *L. japonicus* for symbioses to be restored (Venkateshwaran et al., 2012). The authors propose that DMI1 could derive from POLLUX by one critical amino acid replacement conferring reduced conductance with a long open state, making DMI1 sufficient by itself for symbiosis. Thus, POLLUX could have undergone a first functional specialization step to adapt to nodulation constraints in legumes, and then subsequent specialization within the legume family would have led to DMI1 taking over the role of both POLLUX and CASTOR in *M. truncatula*.

110.4 DECODING OF THE CALCIUM SPIKING

M. truncatula dmi3 mutants cannot establish symbiotic interactions with their microsymbionts (Catoira et al., 2000), but, in contrast to *dmi1* and *dmi2* mutants, they still show calcium spiking in response to symbiotic signals (Wais et al. 2000). MtDMI3 and its orthologue LjCCaMK are calcium/calmodulin-dependent protein kinases (CCaMKs) (Levy et al., 2004; Mitra et al., 2004), the role of which is to perceive the calcium spikes and activate downstream signaling pathways (see Chapters 54, 59).

Gain-of-function mutations of CCaMK induce spontaneous nodulation without rhizobia (Gleason et al., 2006; Tirichine et al., 2007; see Chapter 54). Some of these gain-of-function CCaMK mutations can fully restore not only nodule organogenesis but also successful infection of rhizobia and AM fungi in *L. japonicus* plants carrying mutations in the CSSP genes required for the generation of calcium spiking, for example, *L. japonicus CASTOR* and *POLLUX* mutants. These results demonstrate the central role of CCaMK and that the CSSP genes upstream of calcium spiking are required solely to activate CCaMK (Hayashi et al., 2010). Because of this key role in the CSSP, it has been proposed that MtDMI3/LjCCaMK could act as a switch, distinguishing bacterial and fungal microsymbionts and triggering respective downstream signaling pathways appropriately (Levy et al., 2004; see Chapter 59). This supposes that rhizobia and AM fungi can induce calcium spiking responses with different patterns and, according to the nature of the specific calcium signature, MtDMI3/LjCCaMK would trigger either the nodulation or the mycorrhization genetic program via the activation of specific targets. According to this hypothesis, the CCaMK of legume plants would have evolved to acquire properties that are absent in the orthologous proteins of nonlegumes. As a consequence, it would be expected that orthologues from nonnodulating species such as cereals could restore AM symbiosis, but not nodulation by rhizobia in legume *MtDMI3/LjCCaMK* mutants. To address this question, cross-species complementation experiments were performed using *OsCCaMK*, the rice orthologue of *MtDMI3*, which has been shown to be required for mycorrhizal symbiosis in rice (Chen et al., 2007; Gutjahr et al., 2008). *OsCCaMK* restores mycorrhization (Chen et al.,

2007), but also nodule formation (Godfroy et al., 2006), in *M. truncatula dmi3* mutants. This demonstrates that rice CCaMK can perceive the calcium spiking signal induced by rhizobia and activate the appropriate downstream targets controlling nodule organogenesis. However, restoration of the RL symbiosis is not complete since infection threads were aborted and nodules did not contain bacteria (Godfroy et al., 2006). The inability of *OsCCaMK* to restore rhizobial infection could suggest that this process requires particular functionalities specific to legume CCaMKs. However, the fact that a CCaMK from lily, another nonlegume species, restores both nodulation and rhizobial infection in a *M. truncatula dmi3* mutant (Gleason et al., 2006) is not in favor of such a hypothesis. Furthermore, Banba and coworkers reported that *OsCCaMK* could fully complement symbiosis defects in *L. japonicus ccamk* mutant lines both for AM and RL symbioses (Banba et al., 2008). Thus, the lack of restoration of rhizobial infection by *OsCCaMK* in *M. truncatula* mutants does not reflect the acquisition of a new functionality specific to legume CCaMKs.

This CCaMK has been shown to interact *in planta* with another nuclear-localized protein of the CSSP, called IPD3 (DMI3 Interacting Protein; see Chapter 59) in *M. truncatula* (Messinese et al., 2007) or CYCLOPS (Yano et al., 2008) in *L. japonicus*. As IPD3/CYCLOPS was found to be a substrate of the CCaMK kinase *in vitro*, it has been proposed that the phosphorylation status of MtIPD3/CYCLOPS controls the transcription of infection-related plant genes (Yano et al., 2008). *OsCYCLOPS* from rice was used for cross-species complementation of *L. japonicus cyclops* mutants. Both the AM symbiosis and nodulation by *M. loti* were fully restored, demonstrating that the *CYCLOPS* gene of a nonnodulating cereal can support not only AM fungal but also bacterial endosymbiosis (Yano et al., 2008).

110.5 SYMBIOTIC TRANSCRIPTION FACTORS

The symbiotic pathways intervening downstream of *DMI3* and *IPD3* comprise a set of transcription regulators, including two GRAS-domain transcription factor genes, *NSP1* and *NSP2* (nodulation signaling pathway) (Kalo et al., 2005; Smit et al., 2005; see Chapter 59). These genes were initially reported to be required for the RL symbiosis but not for the AM symbiosis, but in fact, both of them intervene in the CSSP (Delraux et al., 2013; Maillet et al., 2011). Yokota and coworkers demonstrated that *OsNSP1* and *OsNSP2*, the homologues of these genes in rice, are able to fully rescue the RL symbiosis-defective phenotypes of *nsp1* and *nsp2* mutants of *L. japonicus* (Yokota et al., 2010). This functional conservation shows that *NSP1* and *NSP2* from nonlegume plants can fulfill dual regulatory functions, controlling the activation of the RL symbiotic pathway as well as the ancestral role in strigolactone biosynthesis (Liu et al., 2011). Thus, these genes do not appear to have undergone any legume-specific specialization associated with nodulation.

In legume plants, the inactivation of nodule inception (*NIN*), another transcription factor gene, results in the abortion of rhizobial infection (Schauser et al., 1999; see Chapter 59). In contrast to *nsp1* and *nsp2* mutants, this infection defect is not rescued in *L. japonicus nin* mutants by the introduction of *OsNLP1*, the closest homologue of *NIN* in rice (Yokota et al., 2010). These results indicate that the *LjNIN* gene results from the recruitment and specialization of a preexisting gene, and since *NIN* is apparently not required for establishment of the AM symbiosis, this recruitment probably did not come from the mycorrhizal signaling pathway.

110.6 DISCUSSION

Although rice is a monocot plant species phylogenetically distant from legumes, it seems to possess the counterparts of all the legume CSSP genes. In rice as in legumes, inactivation of these genes results in the loss or the alteration of the symbiotic interaction with AM fungi. These observations support the hypothesis that part of the AM symbiotic signaling pathway has been recruited by legumes for nodulation with rhizobia (see also Chapter 108).

Surprisingly, the nodule organogenesis process, which represents a major innovation of the RL symbiosis compared to the AM symbiosis, does not seem to have required any specialization of the CSSP genes, since this process can be fully restored in legume mutants by the corresponding homologous genes from rice. Thus, the acquisition by legume plants of the ability to form nodules could result from the coupling of this signaling pathway with the downstream element *MtCRE1/LjLHK1*, a cytokinin receptor histidine kinase playing an essential role in nodule organogenesis (Gonzalez-Rizzo et al., 2006; Tirichine, et al. 2007).

In contrast to nodule organogenesis, colonization of root tissues occurs in both AM and RL symbioses through infection processes which share common characteristics. Both microsymbionts invade the cells of the root cortex where they develop intracellular structures allowing metabolic exchanges with the plant host. It seems that the ancient exocytotic pathway forming the periarbuscular membrane compartment in the AM symbiosis has been recruited for the symbiosome formation in the RL symbiosis (Ivanov et al., 2012). In spite of these similarities shared by the two symbioses, rhizobial infection cannot be fully rescued by the homologous rice genes in some legume mutants of the CSSP. This is the case for the *OsDMI2/SYMRK* gene that restores nodule organogenesis, but not bacterial infection and symbiosome formation. For *DMI2* genes, only those

versions possessing three LRR domains are able to support the constraints relative to rhizobial infection. Similarly, these constraints could have led to a specialization of the *POLLUX* gene in legume species. Since the roles of these two genes are solely to activate CCaMK via the generation of calcium oscillations (Hayashi et al., 2010; Madsen et al., 2010; see Chapters 54, 57), a parallel specialization of legume CCaMKs would have been expected. However, CCaMKs from nonlegume species are generally able to perceive rhizobium-induced symbiotic signals and to activate downstream targets.

This apparent absence of specialization of DMI3/CCaMK makes it unlikely that legumes can discriminate between rhizobia and fungi on the basis of specific calcium signatures. Furthermore, whereas early observations suggested that different calcium signatures were generated between the RL and the AM interactions (Kosuta et al., 2008), a more recent work using a cameleon calcium sensor showed that the spiking pattern induced in *M. truncatula* during infection by AM fungi or by rhizobia was highly similar (Sieberer et al., 2012; see Chapter 57). However, there are technical limitations in the analysis of calcium spiking that make it difficult to determine whether rhizobia and AM fungal-induced calcium oscillations have exactly the same profiles or not (Sieberer et al., 2012; Singh and Parniske, 2012). Thus, it cannot be ruled out that the two microsymbionts generate slightly different calcium signatures that can be distinguished by legume CCaMKs, which consequently open the appropriate gate to the microsymbiont. In contrast, it can be speculated that CCaMKs from nonlegumes would not have acquired the ability to discriminate between the AM and the RL calcium signatures. This hypothesis could be tested by determining whether legume *ccamk* mutants complemented with *OsCCaMK* are able to selectively trigger the appropriate genetic program or activate both symbiotic pathways, whatever the nature of the microsymbiont.

Alternatively, information on the nature of the microsymbiont could be provided to the host plant by one or more parallel signaling pathway(s), as proposed by Hayashi and coworkers (Hayashi et al., 2010; Madsen et al., 2010). This parallel pathway, though not involving calcium spiking, could nevertheless rely on differential activation of CCaMK through its regulatory domains. It has indeed been shown that truncated variants of CCaMK lacking the autoregulatory domain and the EF hands activate both AM and RL specific genes in the absence of symbiotic partners, whereas autoactive full-length forms of *L. japonicus* CCaMK solely induced nodulation-specific gene expression patterns and no AM-specific genes (Shimoda et al., 2012; Singh and Parniske, 2012; Takeda et al., 2012). Thus, the presence of the autoregulatory domain seems important to define the identity of the downstream pathway. Whether the ability to discriminate between bacterial and fungal parallel pathways reflects a specialization of legume CCaMK, regulatory domains could be studied by complementation experiments using cross-species chimerical CCaMK constructs.

The fact that some CSSP genes are functionally conserved for nodule formation but not for rhizobial entry is a new illustration of the very strict genetic control of rhizobial infection. This stringent genetic control of rhizobium entry in root tissues was previously reported by Ardourel and coworkers (Ardourel et al., 1994), who showed that rhizobial mutants producing modified Nod factors were not able to penetrate the root tissues, but still induced nodule formation.

Similarly, experimental evolution experiments showed that nonsymbiotic bacteria acquire more easily the ability to induce nodule formation on a legume host than the ability to infect this host (Marchetti et al., 2010).

In spite of this tight control of microbial infection by the host plant, the fact that most of the genes of the CSSP have a functional counterpart in rice suggests that engineering cereals to form nodules hosting nitrogen-fixing rhizobia might not be an unrealistic goal, provided that key components missing in rice are identified (see Chapters 108, 109).

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Chapter 111

Occurrence and Ecophysiology of the Natural Endophytic *Rhizobium*–Rice Association and Translational Assessment of Its Biofertilizer Performance within the Egypt Nile Delta

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111.1 INTRODUCTION

111.1.1 Historical Perspective of the Discovery of the Natural Rhizobia–Cereal Association

In the mid-1990s, we began a collaborative research project on novel, beneficial plant–microbe associations involving major cereal crops (Yanni et al., 1995). We wanted to assess their potential for promoting crop productivity with the eventual development of new, improved biofertilizers. Our work focused originally on rhizobia and rice, with a more recent expansion that has included the rhizobia–wheat association.

Our guiding hypothesis was that natural endophytic associations between rhizobia and cereal roots would most likely occur where these cereals are successfully rotated with a legume crop that could enhance and sustain the soil population of the corresponding rhizobial symbionts. Such natural *Rhizobium*–cereal associations would be perpetuated if they were mutually beneficial. If this hypothesis were correct, the cereal roots growing at these sites should harbor, along with other microbes, populations of endophytic rhizobia that are already highly competitive for colonization of the interior habitats of crop roots, being protected from stiff competition with other soil–rhizosphere microorganisms under field conditions. Establishing an endophytic niche is a strategic ecophysiology because a more rapid and intimate metabolic exchange is possible within host plant tissues rather than amidst a larger diverse community at the root epidermal–soil interface.

An ideal place to test this hypothesis was in the Egypt Nile Delta. For more than seven centuries of recorded history, rice there has been rotated with the forage legume Egyptian berseem clover (*Trifolium alexandrinum* L.). In this area, Japonica and (more recently) Indica and hybrid rice cultivars

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are cultivated by transplantation in flooded rice fields, a practice also referred to as "lowland rice cultivation".

An interesting enigma for this conventional farming management is that the clover rotation with rice can replace 25–33% of the N-fertilizer amount recommended for optimal rice production when rice follows a leguminous rather than nonleguminous crop. N-balance data accumulated over decades on Egypt rice farming systems indicated a repeated misconception statement that related the benefit of rice rotation with clover solely to increases in available soil N contributed by mineralization of the N symbiotically fixed, N-rich clover roots and root-nodule residues that are left after the clover's last cut. However, N-balance calculations do not support this explanation. This raised the possibility in our minds that the rhizobial symbiont of clover (*Rhizobium leguminosarum* bv. trifolii) may participate in a natural endophytic association with rice and possibly contribute to this added benefit of the rotation.

111.1.2 Confirmation of the Natural *Rhizobium*–Rice Association

We undertook an ecological approach that involved multiple cycles of field and laboratory studies to detect, enumerate, and isolate rhizobial endophytes from surface-sterilized roots of field-grown rice (Yanni et al., 1997, 2001). Rice plants were sampled at five different field sites during two seasonal rotations with berseem clover in the Nile Delta. The first field sampling was from vegetative regrowth of residual **ratoon** rice surviving rice harvest and grew intermingled among clover plants that followed rice in the same field area in which clover seeds were broadcasted on the soil surface without tillage after rice harvest. The majority of farmers do that to save costs of clover cultivation, with an additional benefit that the first clover cut will contain a balanced mixture of the N-rich clover plant plus some residual cellulose-rich ratoon rice. The second sampling of rice roots was from four different sites in flooded fields of transplanted rice during the next rice growing season. For both samplings, the roots were washed and surface-sterilized until bacteria on the root surface could no longer be cultured. Diluted macerates of the surface-sterilized roots were then inoculated directly on axenic seedling roots of berseem clover growing on N-free medium in closed glass tubes under gnotobiotic conditions. After one month of clover growth in the greenhouse, the inoculated clover plant replicates were examined for root nodulation, and their positively nodulated replicates were scored and subjected to most probable number (MPN) calculations.

This simple experimental design took advantage of the strong positive selection provided by the clover **trap** host to detect and enumerate any endophytic rice root inhabitants of clover-nodulating rhizobia that are naturally present among other microbial endophytes that survived surface sterilization of the rice root and to isolate the numerically dominant strains from nodules of clover plants inoculated with the highest dilutions in the MPN plant infection test. The results from all five sample sites provided a solid confirmation of our guiding hypothesis that clover-nodulating rhizobia do intimately colonize the rice root interior in those fields of the Egypt Nile Delta (Yanni et al., 1997, 2001). The MPN analysis indicated that the population density of clover-nodulating rhizobia was 2–3 logs higher inside the roots of the ratoon rice growing among the clover plants than inside the transplanted rice roots growing in the flooded fields. These results suggested that rice root interiors provide more favorable growth conditions for rhizobia when present in close physical proximity to clover in nonflooded, more aerobic fields. This highlighted a long-term benefit of the rice–legume rotation in promoting this intimate plant–microbe association.

Next, we created a culture collection of pure culture isolates of these rhizobial nodule occupants representing the numerically dominant rhizobial endophytes of rice roots. We verified that they were authentic strains of *R. leguminosarum* bv. trifolii by confirming their ability to nodulate berseem clover in gnotobiotic culture, expressing and demonstrating their N_2-fixing activities on their natural clover host, and comparing the complete nucleotide sequence of their 16S rRNA-encoding gene with other bacteria (Yanni et al., 1997, 2001). The plant tests indicated both effective and ineffective biological N_2-fixing isolates. This was followed by fulfilling stringent tests of molecular Koch's postulates using BOX-PCR genetic fingerprinting and plasmid profiling methods, proving that pure cultures of selected isolates of rice root inhabitant rhizobia can invade rice roots under gnotobiotic culture conditions and be isolated back into pure culture with proof that they are the same authentic inoculant strains (Yanni et al., 1997).

The strain diversity of the isolates was evaluated to gain a better understanding of the breadth of this ecological niche for rhizobia and to guide us in selecting isolates that can represent the genomic diversity of our culture collection for various studies on this association (Yanni et al., 2001). The molecular fingerprint methods indicated that our culture collection of rice-adapted rhizobia contained ten different strain genotypes, representing sufficient variation to define their range in ability to evoke growth responses in rice. It also indicated that diverse populations of *R. leguminosarum* bv. trifolii colonize rice root interiors in flooded and nonflooded rice soils under the rotation of rice and berseem clover in the Nile Delta (Yanni et al., 2001).

Initially by serendipity and later by direct experimentation, we found that some of the rice endophyte strains of clover rhizobia would promote the growth of rice (Yanni et al. 1997, 2001). These results under microbiologically controlled conditions in the laboratory were corroborated by field inoculation trials in the Nile Delta (Yanni et al.

1997, 2001). It became clear that the rhizobial root-nodule symbiont of clover does participate in a natural, beneficial endophytic association with rice without root-nodule formation or biological N_2 fixation.

Since that novel discovery, many tests of the generality of our original finding have indicated that the natural *Rhizobium*–cereal association is much more widespread than just for the rice–clover rotation found and described in the Nile Delta. Following our initial publication, other examples of rhizobia forming natural endophytic associations with nonlegume hosts in rotation with legumes have been reported, including rhizobial associations with wild and domesticated rice, wheat, maize, barley, sorghum, and millet that are growing in agronomic rotations with legumes in Senegal, Venezuela, Canada, Mexico, Morocco, Kenya, India, Pakistan, China, and elsewhere (Chaintreuil et al., 2000; Dazzo et al., 2000; Matiru et al., 2000; Gutierrez-Zamora and Martinez-Romero, 2001; Hilali et al., 2001; Tan et al., 2001; Lupway et al., 2004; Matiru and Dakora, 2004; Mishra et al., 2004; Singh et al., 2006; Mano and Morisaki, 2008; Mishra et al., 2008; Peng et al., 2008; Sun et al., 2008; Singh et al., 2009; Jha et al., 2009; Y. Jing, personal communication; Prabhat Jha, personal communication). In addition to these, other reports also confirm that rhizobial inoculation can provide growth-promoting benefits on rice and other nonlegume crops through various direct and indirect mechanisms (Haque et al., 1993; Höflich et al., 1994; Chabot et al., 1996; Schloter et al. 1997; Webster et al., 1997; Antoun et al., 1998; Prayitno et al., 1999; Antoun and Prévost, 2000; Biswas et al., 2000a; Biswas et al., 2000b; Yanni et al., 2001; Perrine et al., 2001; Hafeez et al., 2004; Chi et al., 2005; Afzal and Bano 2008; Mehboob et al., 2012; Prabhat Jha, personal communication). Thus, rhizobia are now well known for their ability to occupy a third agriculturally important ecological niche as endophytic colonizers of cereal roots and can promote their growth in agroecosystems that host legume–cereal rotations. This creates a *novel triangle of rhizobia's life cycle and natural history* (Figure 111.1) that replaced the textbook version of a mutual 2-component life cycle that contains only the free-living saprophytic growth state (Gram-negative non-N-fixing short rods) in soil outside the roots of the natural legume host, followed by the N_2-fixing endosymbiotic bacteroid state inside the infected legume host cells within the root nodules.

Although originally met with skepticism, there is no longer any scientific basis to doubt that the natural relationship exists, opening the field for other investigators to assess this endophytic association between many other rhizobial strains belonging to different species and various varieties of different cereal crops growing on all continents worldwide that have agroecosystems containing cereal–legume rotations.

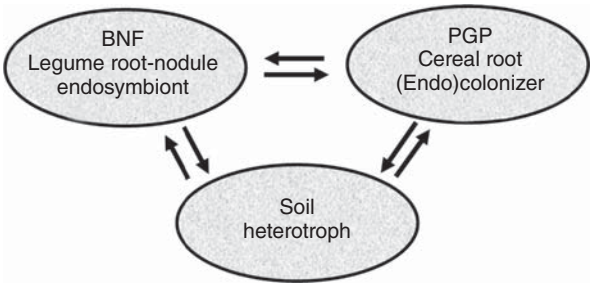

Figure 111.1 Three-component natural lifestyles of ecological niches for rhizobia. Note the newly added niche of plant growth-promoting cereal root (endo)colonizer.

111.1.3 Initiation of Multinational Collaboration Network

By sharing our strains, we established a major multinational network of investigators to cooperate in the creation of a broad-based understanding of the physiology of plant growth promotion (PGP) by our strains and the plant–microbe ecology of this rhizobia–rice association. We envision that the most important long-range impact of this research will be the development and implementation of new microbial biofertilizers that can reduce the major real-world constraint of limited biologically available nitrogen to increase rice productivity in an environmentally friendly way (see Chapters 108, 109). These contributions advanced scientific knowledge on beneficial plant–microbe associations, inevitably assisting marginal low-income farmers who produce rice on N deficit soils that are mostly accompanied by inavailability of many other nutrients, in addition to benefiting manufacturers of agricultural biofertilizer inoculants in the private sector.

111.1.4 Ecophysiology of the *Rhizobium*–Cereal Association

Questions that have interested us most are: what developmental stages of this association are important to provide the enhancement effect and nature of the mechanism(s) that control this enhancement process, and most importantly, what is this N-fixing legume symbiotic micropartner doing on and inside rice roots? Answers to these questions were completely unknown as we began to explore what is happening in this beneficial association.

111.1.4.1 Colonization and Infection. Microscopy has helped to define the infection process in the *Rhizobium*–cereal association. Early studies on the colonization of rice roots by azorhizobia indicated the "crack entry" mode of primary host infection in natural wounds of separated epidermal cells and fissure sites where lateral roots have emerged, followed by colonization within intercellular spaces and host cells of the outer root cortex (Reddy et al., 1997).

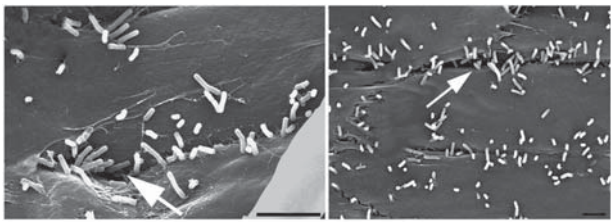

Figure 111.2 Scanning electron micrographs illustrating two examples of crack entry (arrows) of a wild-type endophytic strain of *Rhizobium leguminosarum* bv. trifolii between root epidermal cells of rice. Bar scales equal 10 μm.

The width of these portals of entry can vary considerably but only needs to be ~1 μm for vegetative rods of rhizobia. Two examples of crack entry by an endophytic strain of rhizobia are provided in Figure 111.2. Transmission electron microscopy of well-preserved rice root tissue provided the definitive evidence that confirmed with high magnification/resolution the endophytic colonization of rhizobia within dead host cells adjacent to intact cells of the root cortex (Reddy et al., 1997). Other studies using selected strains of our rice-adapted rhizobia tagged with the green fluorescent protein (Gfp) indicated the crack entry of these bacteria at sites of lateral root emergence, followed by their intercellular multiplication within lateral rootlets and also their ascending migration from these sites of primary host infection to form long fluorescent rows of bacteria inside roots (Prayitno et al., 1999).

More recently, comprehensive microscopy studies of rice plants inoculated with multiple *gfp*-tagged rhizobial strains indicated that the infection process in this association is very dynamic, beginning with bacterial colonization of the rhizoplane especially at lateral root emergence and crack entry into the root interior through separated epidermal cells, followed by *endophytic ascending migration* of the bacteria from the roots up into the stem base and leaf sheath and within lower leaves where they multiply transiently to high local population densities (Chi et al., 2005). *In situ* image analysis indicated that *local* endophytic population densities commonly occur at $\sim 10^8$ per cm^3, but are present in some infected rice tissues at local densities as high as 9×10^{10} rhizobia per cm^3. Thus, this endophytic plant–microbe association is far more invasive than previously thought and includes internal colonization of rhizobia in both belowground and aboveground host tissues. Comparisons of viable plate counts versus direct microscopy indicate that these *gfp*-tagged bacteria remain active for long periods within the rice plant and continue to promote plant growth even after their culturable population densities have declined (Chi et al., 2005).

We are using quantitative computer-assisted microscopy to evaluate spatial patterns of rhizobial distribution on rice roots grown in gnotobiotic culture and under field inoculation conditions in order to better understand the dynamics of their early colonization behavior during this infection process. Our first approach has involved scanning electron microscopy of rice roots inoculated with a candidate biofertilizer strain of *R. leguminosarum* bv. trifolii followed by quantitative image analysis of their spatial patterns of colonization at single-cell resolution using our Center for Microbial Ecology Image Analysis System (CMEIAS©) software (Liu et al. (2001)) and continuously developed thereafter (Dazzo, 2010; Gross et al., 2010; Dazzo, 2012; Dazzo and Yanni, 2013). The results commonly reject the null hypothesis that the spatial pattern of rhizobial colonization of rice is due to random chance, with statistical p values being as low as 10^{-5}. Instead, our spatial ecology analyses indicate that rhizobia form aggregated spatial patterns on the rice rhizoplane, reflecting their highly structured colonization behavior involving cooperative cell–cell interactions of *in situ* ecophysiology (Dazzo et al., 2013; Dazzo and Yanni, 2013). This outcome is a sign of the elegantly symphonic nature of rhizobial interactions during their early colonization of rice roots that ultimately results in this beneficial plant–bacteria association of significant agricultural importance.

111.1.4.2 PGP of Rice by Rhizobial Endophytes.

Our early studies on the natural, endophytic rhizobia–rice association indicated that some strains promote the shoot and root growth of certain rice varieties in gnotobiotic culture (Yanni et al., 1997). Later, more extensive tests established the range of growth responses of Japonica, Indica, and hybrid rice varieties from Egypt, the Philippines, the United States, India, and Australia when these cultivars were inoculated with various rhizobial inhabitants of rice roots. As briefly mentioned above, the results indicated that the diverse rhizobial endophytes evoked a full spectrum (positive, neutral, and sometimes even negative) of growth responses in rice, often exhibiting a high level of strain/variety specificity (Prayitno et al., 1999; Biswas et al., 2000a, 2000b; Yanni et al., 1997, 2001). On the positive side, a chronology of Pgp^+ responses of rice to rhizobia manifests as increased seedling vigor (faster seed germination followed by increased root elongation, shoot height, leaf area, chlorophyll content, photosynthetic capacity, root length, branching, and biomass). Figure 111.3 illustrates the aboveground growth response of rice to inoculation with *R. leguminosarum* bv. trifolii and a low dose of supplemental N-fertilizer and, most importantly, the synergistic positive growth response when both treatments are included that extends beyond either treatment alone. These growth-promoting responses carry over to produce increased yield and N content of the straw and grain at maturity. More information on these synergistic benefits of significant agronomic importance is covered later.

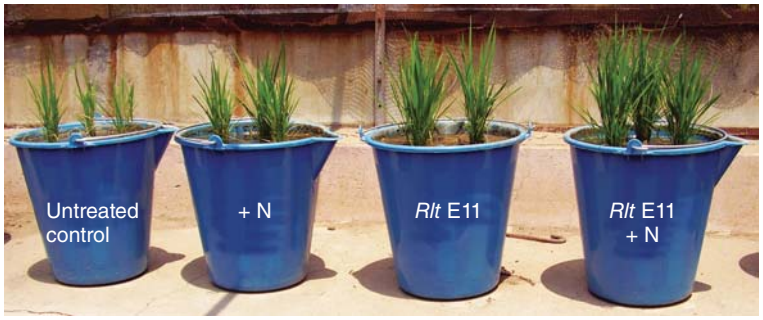

Figure 111.3 Pot experiment showing early, aboveground growth response of the *Giza-178* variety of rice to inoculation with *R. leguminosarum* bv. trifolii with and without a low-dose (48 ppm N) supplemental N-fertilizer treatment. Note the outstanding positive growth response of rice when receiving both treatments. This synergistic result recurs repeatedly in field inoculation experiments.

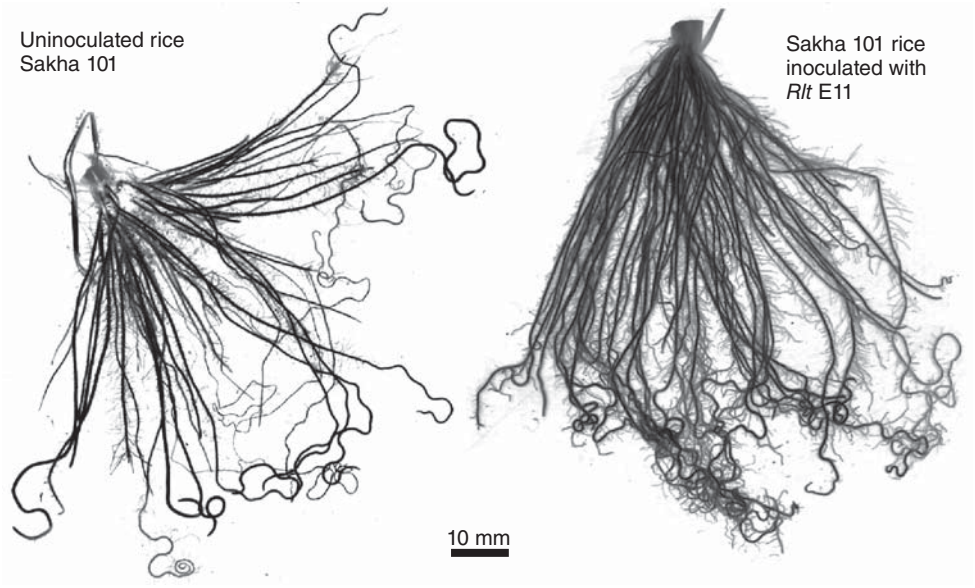

Figure 111.4 Architecture of Sakha-101 rice roots after gnotobiotic growth without (a) and with (b) *R. leguminosarum* bv. trifolii inoculation.

111.1.4.3 Underlying Mechanisms of PGP. The ability of some endophytic rhizobial strains to promote the growth of rice prompted follow-up studies to identify possible mechanisms operative in this beneficial plant–microbe interaction. These studies have addressed the following possibilities: (i) rhizobial induction of an expansive root architecture with enhanced efficiency in plant mineral nutrient uptake; (ii) rhizobial production (or host activation) of extracellular growth-regulating phytohormones; (iii) rhizobial solubilization of precipitated inorganic and organic phosphate complexes, thereby increasing the bioavailability of this important plant nutrient; (iv) associative nitrogen fixation by rhizobia; and (v) rhizobial production of siderophores that increase the bioavailability of iron.

Although biological control of phytopathogens (via direct antagonism or induction of systemic disease resistance) is traditionally recognized as another important mechanism of PGP by rhizobacteria (Kloepper et al., 1980; Haque and Ghaffar, 1993), we have not tested whether this mechanism is operative in our system because our rhizobial inoculants can promote rice growth in gnotobiotic culture (independent of phytopathology) and because the (already blast-resistant) uninoculated control plants lack any disease symptoms in our field inoculation trials in Egypt (Yanni and Dazzo, unpublished observations).

Stimulation of Root Growth and Nutrient Uptake Efficiency. The responsive rice varieties commonly develop expanded root architectures when inoculated with selected biofertilizer strains of rhizobia. An example is presented in Figure 111.4.

This suggests that these rhizobial endophytes alter root development in ways that could make them better "miners" more capable of exploiting a larger reservoir of plant nutrients from existing resources in the rhizosphere soil. This possibility was suggested in early studies showing significantly increased production of root biomass in plants that had been inoculated (Prayitno et al., 1999; Biswas

et al., 2000a; Yanni et al., 1997, 2001) and by studies using greenhouse potted soil indicating significant increases in N, P, K, and Fe uptake by rice plants inoculated with selected rhizobia, including our test strains (Biswas et al., 2000b). More recent studies have confirmed this hypothesis using plants grown gnotobiotically with rhizobia in nutrient-limited medium (50% Hoagland's), followed by measurements of root architecture and mineral nutrient composition using image analysis and atomic absorption spectrophotometry, respectively (Yanni et al., 2001). In these latter studies, inoculated plants developed a more expanded root architecture and accumulated higher concentrations of N, P, K, Ca, Mg, Na, Zn, and Mo than did their uninoculated counterparts.

Interestingly, the levels of Fe, Cu, B, and Mn were not statistically different in the same inoculated versus uninoculated plants under these microbiologically controlled experimental conditions (Yanni et al., 2001). This selectivity in terms of which plant nutrients exhibit increased accumulation as a result of inoculation argues against an across-the-board general enhancement of mineral accumulation due just to expanded root architecture with increased absorptive biosurface area. The results indicate, quite interestingly, that bacteria can modulate the rice plant's plasticity that enables it to control the adaptability of its root architecture and also the physiological processes for more efficient acquisition of selected nutrient resources *when they become limiting*. This same mechanism is largely responsible for the beneficial growth promotion effect of *Azospirillum brasilense* on grasses (Tien et al., 1979; Umali-Garcia et al., 1980; Okon and Kapulnik, 1986; Bashan et al., 1990; Okon and Labandera-Gonzalez, 1994; see Chapter 90). Rhizobacteria can thus control the ecophysiology of mineral nutrient uptake by plant roots.

These *Rhizobium*-induced increases in the accumulated mineral composition of rice plants raise new possibilities regarding their potential impact on the human nutritional value of this world's most important crop. A potential value-added benefit resulting from rhizobial inoculation is their ability to increase the nutritional value of resulting rice grain, not only for increased N (mostly in the form of storage protein) but several other macro- and micronutrients as well. For instance, rice is an important and indeed even the major bioavailable source of some essential minerals, for example, zinc, in the human diet, particularly in developing countries (IRRI, 1999). Zinc is considered an essential supermicronutrient that is important for the maturation of the reproductive organs in women and the developing fetus. Our rhizobial inoculants have the capacity to increase the zinc content of rice grain in the field (Yanni et al., 2001).

Enhanced efficiency in the uptake of nutrient resources by inoculated rice could be a two-edged sword if accompanied by their enhanced bioaccumulation of toxic heavy metals. We, therefore, analyzed other rice grain samples from this same field inoculation experiment for their heavy metal content (Hg, Se, Pb, Al, and Ag). The results indicated no significant differences in the low levels of these toxic heavy metals in the rice grain of uninoculated versus rhizobial endophyte-inoculated treatments (Yanni et al., 2001).

SDS-PAGE and RP-HPLC analyses of the protein composition of field-grown Giza-177 rice grains indicated no discernible differences in the *ratios* of their major nutritionally important storage proteins, particularly glutelin, albumin, and globulin, as a result of inoculation with the rhizobial endophyte strain E11 (Yanni et al., 2001). Field inoculation with this rhizobial endocolonizer thus did not qualitatively alter rice grain protein, as all nutritionally important proteins were present in the treated and control samples in similar ratios. However, since inoculation with rhizobia causes a significant increase in total grain nitrogen per hectare of crop (in protein form), the benefits of inoculation to small farmers will include an increase in the *quantity* of rice grain protein produced per unit of land used for cultivation. This increases the nutritional value of the harvested grain as a whole in comparison with uninoculated rice produced.

Considered collectively, these studies indicate that rice plants inoculated with our rhizobial biofertilizers produce rice grain whose human nutritional value is equal to or improved (depending on the nutrient considered) as compared to uninoculated plants.

Secretion of Plant Growth Regulators. Early studies suggested that rhizobial endophyte strain E11 produced the auxin indole acetic acid (IAA) in pure culture and in gnotobiotic culture with rice (Biswas et al., 2000a, 2000b), as has been observed with *Azospirillum* (see Chapters 90, 91). Further studies indicated that production of IAA equivalents by strain E11 was tryptophan dependent. We developed a simple defined medium to optimize production of IAA by fast-growing rhizobia, and axenic bioassays of filter-sterilized culture supernatant from strain E11 grown in this defined medium showed an ability to stimulate rice root growth (Yanni et al., 2001). These results suggest that endophytic strains of rhizobia can boost rice growth by producing extracellular bioactive metabolites that promote the development of more expansive root architecture.

These results logically led us to identify the growth-regulating phytohormones produced and secreted by strain E11 in pure culture. Analysis of its culture supernatant using electrospray ionization/gas chromatography/mass spectrometry indicated the presence of IAA and a gibberellin (consistent with GA_7) (Yanni et al., 2001). These represent two different major classes of plant growth regulators that play key roles in plant development.

Rhizobial Solubilization of Precipitated Phosphate Complexes. Most Nile Delta soils used for rice cultivation contain around 1000 ppm phosphorus, primarily in the

unavailable form of precipitated tricalcium phosphate [$Ca_3(PO_4)_2$]. Although the submergence conditions normally prevail in rice fields, less than 8 ppm (Olsen) P is available to rice. Therefore, any significant solubilization of precipitated phosphates by rhizobacteria *in situ* would enhance phosphate availability to rice in these soils, representing another possible mechanism of PGP for rice under these field conditions. We tested the diversity of rice-adapted rhizobia for phosphate-solubilizing activity on culture media impregnated with insoluble organic and inorganic phosphate complexes. Our improved, double-layer plate assay indicated that some strains are active in solubilizing insoluble P complexes, both the inorganic form (calcium phosphate) that normally dominates the rice rhizosphere area and the organic form (inositol hexaphosphate = phytate) that accumulates inside the root system (Yanni et al., 2001). These positive results indicate the ability of rhizobial inoculants to perform extracellular acidification that can solubilize the inorganic phosphate complex(es) in the rhizosphere and also produce phosphatase enzymes (phytase) for solubilization of the stored organic forms accumulated inside the root tissue during the early stages of plant growth. This extracellular PGP^+ activity would potentially increase the availability of phosphorus for rice in its nutrient-depletion zone of rhizosphere soil and thereby promote rice growth when soil phosphorus is limiting.

Associative Nitrogen Fixation. Rice plants accumulate more N-shoot and N-grain when inoculated with selected strains of rhizobial endophytes (Biswas et al., 2000b; Yanni et al., 1997, 2001). However, this additional combined nitrogen is mainly derived from soil mineral N rather than from BNF. This conclusion is based on several lines of evidence:

1. The growth benefits to rice by rhizobial inoculation are enhanced rather than suppressed when N-fertilizer is provided (Prayitno et al., 1999; Biswas et al., 2000a, 2000b; Yanni et al., 1997, 2001; Yanni and Dazzo, 2010). This contrasts with the well-known suppressive effect of mineral N application on development of the N_2-fixing *Rhizobium*–legume root-nodule symbiosis.
2. The degree of rice growth benefit linked to inoculation with rhizobial endophyte biofertilizers does not correlate with their degree of nitrogen-fixing activity when in symbiosis with their normal legume host (in our case, the berseem clover), since some rice endophyte strains of rhizobia that are Fix-minus on berseem clover are nevertheless Pgp^+ on rice (Yanni et al., 2001), and therefore, field inoculation with those N fix^- forms is purposely avoided to prevent negative agronomic consequences.
3. Acetylene reduction tests for BNF did not detect associative nitrogenase activity on rice plants whose growth had been promoted gnotobiotically by inoculation with rhizobial endophytes (Yanni et al., 1997; Biswas et al., 2000a).
4. Greenhouse studies using the ^{15}N-based isotope dilution method indicated that most of the increased N uptake in inoculated plants is not derived from BNF (Biswas et al., 2000b).
5. Measurements of the natural abundance of nitrogen isotope ratios ($\delta^{15}N$) on field-grown plants indicated that their greater proportion of N resulting from inoculation with rhizobial strain E11 is not derived from BNF (Yanni et al., 2001). The proportion of rice plant N derived from BNF was approximately the same (~10%) with versus without inoculation.

Considered collectively, these results indicate that biological nitrogen fixation is not a major mechanism responsible for the significantly positive growth response of selected rice cultivars to inoculation with these rhizobial endophytes of rice.

Production of Fe-Chelating Siderophores. Siderophore production potentially provides a dual mechanism of PGP: enhancing bioavailability and uptake of Fe for the plant and suppression of rhizosphere pathogens unable to utilize the Fe–siderophore complex. However, none of the genotypes of rhizobial endophyte strains in our culture collection produced detectable siderophores (Yanni et al., 2001), so this mechanism seems not to be applicable for them. Nevertheless, other rhizobial strains may enhance Fe uptake in rice (Biswas et al., 2000a).

111.1.4.4 Proteomic Analysis of Rice Inoculated with Rhizobia. Proteomic analysis has provided fundamentally new information showing that inoculation of rice plants grown gnotobiotically with certain beneficial, endophytic rhizobia induces the production of identifiable plant proteins that contribute to better plant performance and health (Chi et al., 2010). Energy-associated proteins that enhance both light and dark reactions of photosynthesis in chloroplasts are upregulated in leaf sheathes and leaves. Proteins involved in phytohormone metabolism (e.g., IAA biosynthesis) are upregulated in aboveground plant tissues and downregulated in roots. Several defense-related proteins are upregulated in root and shoot tissues. Considered collectively, these plant molecular biology results predict that certain endophytic rhizobia promote rice growth by (i) enhancing photosynthesis to build plant biomass more efficiently, (ii) regulating phytohormone status *in planta* to promote vegetative growth, and (iii) activating defense responses to minimize the negative effects of environmental and pathogenic factors.

111.1.5 Extension of *Rhizobium* Benefits to Other Rice Varieties

For agronomical reasons, it is important to examine whether the various desirable interactions of these rhizobial endophytes can be extended to cereal varieties that are preferred by farmers in cropping systems throughout the world. Because many characteristics of this association exhibit high strain/variety specificity, tests of their compatibility at the lab bench are necessary before they are tested in the field. Our studies so far have included rice genotypes commonly used in Egypt (Sakha-101, Sakha-102, Sakha-103, Sakha-104, Giza-175, Giza-177, Giza-178, Giza-181, and Jasmine rice), the United States (M202 and L204), Australia (Calrose and Pelde), and India (Pankaj).

But what about rice varieties preferred by low-income farmers who cultivate rice on marginally fertile soils and who cannot afford to purchase fertilizers? To address this question, a study was done to measure how well the rhizobial endophyte strain E11 can colonize the root environment of four rice genotypes (Sinandomeng, PSBRC 74, PSBRC 58, and PSBRC 18) preferred by Filipino peasant farmers because of their good yielding ability and grain characteristics. Cells were inoculated on axenic seedling roots and then grown gnotobiotically in hydroponic tube culture, and the resultant populations were enumerated by viable plate counts. For comparison, seedlings of equal size received an equivalent inoculum of a local, unidentified isolate BSS 202 from *Saccharum spontaneum* used as a Pgp^+ biofertilizer inoculant for rice in the Philippines.

The results indicated significant colonization potential of strain E11 on roots of all four rice genotypes (Yanni et al., 2001). The implications of this experiment are significant: strain E11 exhibits no obvious difficulty in its potential to intimately colonize roots of not only the "superior" rice varieties that have undergone significant breeding development but require high N inputs for maximum yield but also with other rice varieties preferred by peasant farmers because they perform acceptably on marginally fertile soil without significant N-fertilizer inputs. The latter type of rice production could derive significant benefit from the biofertilizer inoculants that our research is developing.

111.1.6 Translational Research on Rice Biofertilization Using *Rhizobium* under Field Conditions

Our strategy to maximize the benefits of rice biofertilization was to initiate a translational research/technology program for enhancement of its production in the Egypt Nile Delta based on our findings that strains of rice-colonizing rhizobial endophytes positively contributed to higher paddy yields and N-fertilizer use efficiencies. This translates to significantly reduced need for additional N-fertilizer applications for increased rice yields, fully consistent with sustainable agriculture and environmental soundness.

The production of rice up to a level of global food security is currently not possible without major nutrient inputs, especially N. Indirectly, rice is able to utilize a basal level of fixed N through N_2-fixing activities of diverse diazotrophs in its agroecosystem. Our measurement of that process in the Nile Delta using $^{15}N/^{14}N$ natural abundance methods indicates that roughly 10% of the N in mature rice plants is derived from BNF (Yanni et al., 2001). Serious economic and environmental problems associated with excessive use of fertilizer application to enhance rice production could then be mitigated if cereal crops could establish a more direct and intimate association with beneficial bacteria that promote its growth and increase grain yield with less dependence on chemical N-fertilizer input. This natural association can significantly improve rice plant growth, resulting in an increase in its grain productivity and agronomic N-fertilizer use efficiency (kilogram grain yield/kilogram N-fertilizer), making it possible to increase rice grain yields with less dependence on nitrogen fertilizer inputs (Yanni et al., 2001; see Chapter 109).

In situations exemplified by our field experiments at the Kafr El-Sheikh Governorate in Egypt (Yanni et al., 1997, 2001), inoculation of rice with rhizobia followed rather than replaced crop rotation with the berseem clover legume crop. Rice could then gain benefits from both biological associations of rhizobia: indirectly as the N_2-fixing root-nodule occupant of clover and directly as an active plant growth promoter in rice roots. This strategy is intended to augment the anticipatory colonization potential of superior inoculant strains in competition with other rhizosphere microorganisms for endophytic niches in rice above the natural level of inoculum potential carried over from decay of residues of the previous clover crop. The strategy is appealing from the environmental impact standpoint since it makes greater use of the natural resource of beneficial plant–microbe interactions.

We have tested selected rhizobial isolates representing a diversity of strain genotypes in a total of 38 field inoculation trials, with earlier studies conducted on small (20 m^2) replicated plots (Yanni et al., 1997, 2001) and later in trials on large upscaled farmers' field locations in the Nile Delta (Yanni and Dazzo, 2010). These experiments were designed and conducted in areas where rice and legumes have been adopted in crop rotation since the beginning of recorded history. This distinctively enabled us to assess the agronomic performance of rhizobia in rice biofertilization under a broad range of soil characteristics and landscapes and, most importantly, to compare it to real-world, present-day on-farm baselines in rice grain production.

In the same set of experiments, replicate plots were also included to measure how much N-fertilizer application could be replaced and/or how much more grain yield could be obtained from the same field area with application of

biofertilizer inoculants even when N-fertilizer expressed its maximum potential in enhancement of rice growth and performance. The experiments included N-fertilizer application at three ascending quantities: 1/3, 2/3, and the full recommended N-fertilizer dose assessed and recommended by the Agricultural Research Center, Egypt (http://www.arc.sci.eg/), for maximum grain yield without biofertilizer application. The N-fertilizer was added as urea (46% N) in two equal doses at 15 and 30 days after transplantation of the rice seedlings.

Three initial field trials were done in 20 m^2 subplots with four replications. Integrated field management practices are fully described in Yanni et al. (1997, 2001). Results of the three field inoculation trials indicated that some rhizobial strains/rice varieties combinations significantly increased rice grain yield, straw production, agronomic N-fertilizer use efficiency, and harvest index. The results further indicate the potential opportunity to exploit this plant–rhizobia association by developing biofertilizer inoculants that may assist low-income farmers in increasing cereal production with no further additions of chemical N-fertilizer beyond the recommended amount for the rice variety, fully consistent with both sustainable agriculture and environmental biosafety.

Interestingly, rice grain yield in the first experiment (Yanni et al., 1997) negatively responded to excessive N-fertilizer application over the recommended (included for comparison reasons) along with biofertilization. This (unrecommended fertilization practice) results in the well-known phenomenon in rice agronomy called "lodging", which is a consequence of overacquisition of N in plant tissue, leading to excessive vegetation in the later growth stage in which the plant must go to panicle initiation rather than excessive production of vegetative parts, producing semierect shoots that are vulnerable to wind damage and have increased spikelet sterility and production of late nonproductive tillers. This adverse growth response was intensified further by biofertilization that led to overabsorption of available N that exceeded the plant's need to complete its natural life cycle (seed to seed).

Later (Yanni and Dazzo, 2010), we conducted 24 large-scale inoculation trials using our selected endophytic strains of rice-colonizing rhizobia in farmer's fields in nine counties of the Kafr El-Sheikh Governorate located in the north–west–central regions of the Nile Delta (Figure 111.5). The rice cultivation area in this governorate represents approximately 18–25 % of the total area used annually for rice cultivation in Egypt. In this massive field experimentation program, we tested the performances of seven genotypically distinct inoculant strains of *R. leguminosarum* bv. trifolii, used individually or in consortia, for inoculation of five rice varieties along five rice growing seasons (May through October) during 2000, 2002, 2003, 2004, and 2005, in which 1, 4, 5, 6, and 8 experiments were conducted, respectively. The field experimentation directory in

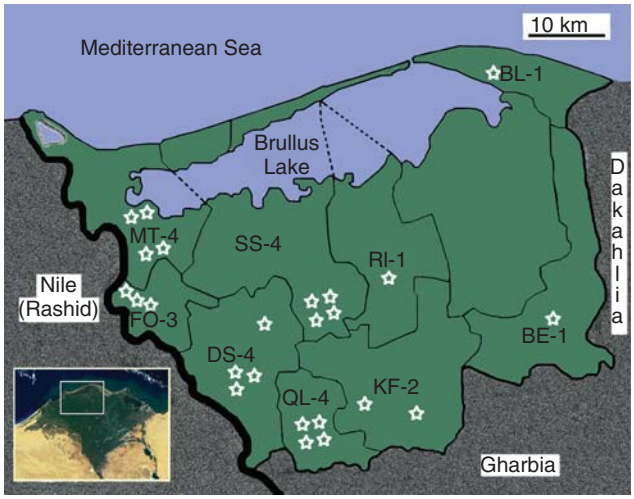

Figure 111.5 Twenty-four sites in nine counties of the Egyptian Nile Delta where large-scale field inoculation trials of the rhizobia–rice association were conducted. County abbreviations are as follows: MT, Metobas; SS, Sidi Salem; RI, Riad; Fo, Fowa; DS, Desouk; QL, Qalein; BE, Biala; BL, Baltem; KF, Kafr El-Sheikh city (including Sakha). The framed insert in the lower left corner is a satellite image showing the portion of the Nile Delta represented by this map.

Table 111.1 presents pertinent information on the location, year, previous crop, rice variety, rhizobial inoculant strain(s), field soil salinity, texture, important chemical properties, and area of the replicated subplots.

111.2 METHODOLOGY

Properties of the used rice varieties, soil sampling, soil tillage, preparation of rice nurseries, transplantation processes, field stand density, amounts, sources, and times of application of phosphatic and potash fertilizers and irrigation regime, experiment follow-up, harvest, yield and yield components, and other agronomic parameters are all described in Yanni and Dazzo (2010). To assess the nitrogen fertilization economy in the presence and absence of bacterial inoculation, three N-fertilizer amounts were placed as the main plot treatments. These 24 large-scale field experiments plus the previously mentioned three small-scale field experiments performed at the experimental farm of the Sakha Agricultural Research Station represent a total of 27 field inoculation trials in which 9 rice varieties and 7 genotypically distinct, indigenous rhizobial endophyte strains were used. Samples for yield comparison were taken from the identical, noninoculated area cultivated with the same rice variety during the same period in the adjacent fields that were managed by the farmer while recalling his own conventional farming practices that he acquired himself or by transfer from his ancestors. We located our experimental plots directly adjacent to the farmer's conducted

Table 111.1 Directory and basic information on the experimental locations that hosted the translational large-scale inoculation trials in farmers' fields in the nile delta

Location		Growth		Previous Crop		Rice Variety		Inoculant Strain(s) *R. leguminosarum* bv. trifolii	
Near city	Expt.	Season	Expt.	Crop	Expt.	Var.	Expt.	Strain	Expt.
Baltem	2	2000	1	Berseem clover	1–3, 6, 9, 12, 13, 15, 18 - 23	Jasmine	1	E11 + E12	1–5
Biala	3	2002	2–5	Wheat	4, 5, 7, 8, 10, 14, 16, 17, 24	Giza-177	8	E24	6, 7
Desouk	8, 15, 19, 21	2003	6–10	Faba bean	11	Giza-178	2–5, 9, 10, 12–14, 17, 18, 20, 24	E39	8 –10
El-Read	16	2004	11–16			Sakha-101	7, 11, 19, 22, 23	E18+E26+E36	11–16
Fowa	9, 12, 18	2005	17–24			Sakha-104	6, 15, 16, 21	E11+E12+E18+E24+E26	17–24
Kafr El-Sheikh	1	Soil salinity (ppm): 0–2500 ppm at 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 19, 20, 21, 22, 23, and 24; 2501–5000 at 1 and 6; 5001–7500 ppm: 7213 at16 and 8609 at location 2							
Metobas	4, 6, 13, 20	Soil textures (http://www.google.com.eg/search?hl=en&q=soil+triangle+diagram&meta=&aq=0&oq=Soil+triangle) were clay at locations 1, 3, 5, 7, 8, 10, 14, 17, 22, 23, and 24; clay loam at 16, 18, and 19; silty clay at 13, 15, 20, and 21; silty clay loam at 4, 6, 9, and 12; and silty loam at 2 and 11							
Qalien	7, 11, 23, 24	Soil chemical analysis (Black et al. 1965, Jackson 1967): pH 7.8–8.1, CaCO3 1.4–5.4 %, and organic matter 1.9 to 2.3 %							
Sakha	22	Replicated field plot area is 20 m^2 for experiments 1–16 and 52.5 m^2 for experiments 17–24							
Sidi Salem	5, 10, 14, 17								

Table 111.2 Mean of paddy yield of the different varieties obtained under supervision of the research team and those of the farmers in their adjacent fields as affected by inoculation and N-fertilization

Rice Variety	Expt.	N (kg N/ha)	Mean of Paddy Yields (ton/ha)		Mean of Increase (%)	Mean of Yields in the Adjacent Farmer's Fields
			Noninoculated	Inoculated		
Jasmine	1	48	7.298	8.641	18.4	7.162
		96	7.775	8.867	14.0	
		144	8.725	9.404	7.8	
Giza-177	8	48	7.176	8.306	15.7	9.520
		96	8.247	9.270	12.4	
		144	9.008	10.008	11.1	
Giza-178	2–5, 9, 10, 12–14, 17, 18, 20, 24	48	8.548	9.708	13.6	10.154
		96	9.943	10.648	7.1	
		144	10.736	11.231	4.6	
Sakha-101	7, 11, 19, 22, 23	48	9.369	9.783	4.4	9.420
		96	9.886	10.695	8.2	
		144	10.294	10.770	4.6	
Sakha-104	6, 15, 16, 21	48	9.524	10.215	7.3	9.546
		96	10.112	10.762	6.4	
		144	10.611	11.102	4.6	

area so that he and we could easily recognize differences in the crop growth features along the cultivation season and also calculate together differences in the gained yield and N-fertilizer use efficiency in the two adjacent field areas.

111.2.1 Grain Yields in Our Scaled-Up Field Experiments Compared to the Farmer's Yields

Table 111.2 indicates the best performance obtained with field inoculation treatments using our strains and the three tested N-fertilization rates on the scaled-up, researcher-supervised experimental plots versus yields of the same variety obtained simultaneously by the farmer using the recommended rate of N-fertilizer (mostly more) without inoculation or supervision by the research personnel. The results indicate the synergistic beneficial effects of rhizobial inoculation and N-fertilization on increased grain yield for all rice varieties. The means of percentage increases due to inoculation over all the 24 field experiments ranged from 4.4% to 18.4%, 6.4% to 14.0%, and 4.6% to 11.1% with application of N-fertilizer at 48, 96, and 144 kg/ha, respectively. The results also indicated how the rice varieties differed in magnitude of response to biofertilization. Maximum rice production of 9.404, 10.008, 11.231, 10.770, and 11.102 ton paddy yield/ha for the rice varieties Jasmine, Giza-177, Giza-178, Sakha-101, and Sakha-104, respectively, were obtained with fertilization by 144 kg N/ha along with biofertilizer application. The corresponding figures obtained without biofertilization were 8.725, 9.008, 10.736, 10.294, and 10.611, respectively, registering mean increases of 0.679, 1.000, 0.495, 0.476, and 0.491 ton/ha attributed to biofertilization. This confirms our earlier results (Yanni et. al., 1997, 2001) that strain/variety specificity is controlling this beneficial cereal–strain endophytic association. Higher percentage increases in rice yields (not necessarily the maximum yields) of the five varieties were obtained using biofertilization along with the 48 and 96 kg N-fertilizer/ha, indicating better crop performance when biofertilization was used along with low rather than high N application doses and that inoculation can significantly reduce, but not completely eliminate, the need for chemical N-fertilizer to accomplish maximum grain yield.

111.2.2 Performance of Biofertilization in Salt-Affected Soils

In Egypt, saline soils are very common in the northern coastal region of the Nile Delta that borders the Mediterranean Sea (Figure 111.5) where we have been conducting this translational research program. Submerged rice is predominantly grown not only for its importance as an economically staple cereal crop but also because reclamation of salt-affected soils in those areas can be facilitated by percolation of Nile freshwater through the saline soil profile, which can wash the high salt content downward to drainage canals.

Data in Table 111.3 report the performance of rhizobial biofertilization in different salt-affected soils. Increasing N application rate resulted in concomitant increases in paddy yield. Maximum yields were obtained by biofertilization along with application of 144 kg N/ha when used in soils having salinity levels of up to 7500 ppm and with only 96 kg N/ha added under a salinity level of 8609 ppm. The means of

Table 111.3 Mean of paddy yields obtained under the different salinity levels dominated the experimental locations as affected by inoculation and N-fertilization, when produced under supervision of the research team and under the farmer's conventional practices in their adjacent fields

Soil Salinity (ppm)	Expt.	N (kg N/ha)	Mean of Paddy Yields (ton/ha)		Mean of Increases by Inoculation (%)	Mean of Yields in the Adjacent Farmer's Fields (ton/ha)	Increase by the Best Experimental Treatment over the Farmer's Yield (%)
			Noninoculated	Inoculated			
0–2500	A	48	9.204	9.680	5.2	9.426	18.0
		96	10.002	10.671	6.7		
		144	10.662	**11.122**	4.3		
2501–5000	B	48	9.763	10.627	8.5	9.946	13.0
		96	9.885	10.815	9.4		
		144	10.519	**11.241**	6.9		
5001–7500	C	48	9.154	9.857	7.6	7.140	46.2
		96	9.826	10.309	4.9		
		144	10.056	**10.439**	3.8		
>7500	D	48	6.959	8.134	16.9	8.330	3.5
		96	6.424	**8.623**	34.2		
		144	6.881	8.305	20.7		

maximum paddy yields obtained with inoculation under the salinity levels (ppm) of up to 2500, up to 5000, up to 7500, and >7500 were 11.122, 11.241, 10.439, and 8.623 ton/ha, obtained using application of 144, 144, 144, and 96 kg N/ha, respectively, comparing to 10.662, 10.519, 10.056, and 6.959 obtained with no inoculation and application of 144, 144, 144, and 48 kg N/ha in the same respective order. Moreover, the comparison between means of yields of the best experimental treatments adopted by the research team and those of the farmers (Table 111.3) indicated 18.0, 13.0, 46.2, and 3.5 % yield increases, respectively.

These results highlight the benefit of biofertilization as the mean of grain yield obtained with inoculation exceeded what could be obtained in the corresponding noninoculated controls under all the soil salinity levels. The percentage increases in grain yield due to inoculation followed a special tendency in which – with few exceptions – it increased proportionally with salinity intensification. The higher soil salinity in the county (Figure 111.5) that includes the northern city of Baltem (experiment #2) most likely contributed to the lower grain yield of the variety Giza-178 obtained in the noninoculated treatments when compared to the higher grain yield of the same rice variety obtained in noninoculated treatments at other locations that had less soil salinity. This indicates that rice was only able to tolerate the high salinity stress when inoculated with and receiving the benefit(s) of the rice-colonizing rhizobia. Interestingly, the mean increase in yields resulting from inoculation was highest at that same location, suggesting that *inoculation of rice with certain endophytic rhizobial strains may help to alleviate the adverse effects of salinity stress on rice grain production*. Similar trends have been previously found for the root-nodule symbiosis between *Bradyrhizobium* spp. with pigeon pea (*Cajanus*) and *Rhizobium loti* with chickpea (Rao and Sharma 1995; Subbarao et al., 1990). This raises the interesting possibility that rhizobial inoculation may help farmers obtain higher rice yields during and just after performing primary reclamation processes to saline soils (as long as the benefit-to-cost ratio is acceptable).

111.2.3 Plant Growth Tendencies and Biomass Production as Affected by Biofertilization

The harvest index (grain yield/aboveground harvested grain plus straw biomass, expressed as a percentage) is an important unitless agronomic parameter for plant breeders. It reflects the portion of plant growth directed to reproductive production of grain versus vegetative production of aboveground shoot and leaf biomass, mostly at the expense of grain production. Calculating this parameter at harvest can indicate whether there was an imbalanced growth due to excessive vegetative growth that consumed large amounts of nutrients rather than mobilized them for grain production. This mostly takes place when the plant was supplemented with excessive unnecessary amounts of N-fertilizer during the panicle initiation and flowering stages. This leads to production of extra nonreproductive tillers (tillers bearing no panicles) with soft plant tissues that are more susceptible and easily invaded by bacterial, viral, and insect pests, leading to decreased grain production from fewer panicles and/or lower grain weight.

The harvest indices in our field experiments (Table 111.4) confirmed that for all three levels of N-fertilizer applications, rice straw at harvest was higher than the corresponding

Table 111.4 Influence of *Rhizobium* inoculation on harvest index at eight locations in the Nile delta

Expt. No.	Rice Var.	N (kg/ha)	Noninoculated Control	Inoculated	Mean	Expt. No.	Rice Var.	N (kg/ha)	Noninoculated Control	Inoculated	Mean
1	Jasmine	48	37.2	**40.3**	38.8[a]	11	Sakha-101	48	46.4	46.0	46.2[a]
		96	34.5	**40.2**	37.4[a]			96	47.2	46.4	46.8[a]
		144	33.5	**37.8**	35.7[a]			144	43.0	42.7	42.9[b]
		Mean	35.1[a]	**39.4[b]**				Mean	45.5[a]	45.0[a]	
2	Giza-178	48	31.1	**33.3**	32.2[a]	12	Giza-178	48	46.1	47.0	46.6[a]
		96	30.7	32.4	31.6[a]			96	43.9	44.3	44.1[b]
		144	30.9	**33.9**	32.4[a]			144	44.9	45.5	45.2[C]
		Mean	30.9[a]	**33.2[b]**				Mean	45.0[a]	45.6[a]	
6	Sakha-104	48	55.0	54.2	54.6[a]	13	Giza-178	48	47.8	46.0	46.9[a]
		96	54.5	53.1	53.8[a]			96	44.6	45.4	45.0[b]
		144	52.4	51.8	52.1[b]			144	43.8	45.6	44.7[b]
		Mean	54.0[a]	53.0[a]				Mean	45.4[a]	45.7[a]	
10	Giza-178	48	41.3	43.6	42.5[a]	14	Giz-178	48	44.2	44.1	44.2[a]
		96	39.7	44.7	42.2[a]			96	42.2	41.6	41.9[b]
		144	38.2	46.3	42.3[a]			144	40.3	40.0	40.2[C]
		Mean	39.7[a]	44.9[b]				Mean	42.2[a]	41.9[a]	

Harvest index: % of grain yield/grain + straw yields. Means superscripted by different letters in the same column (for N-fertilization rates) or highlighted in bold (for inoculation) for each experiment are statistically different at the 95% confidence level.

noninoculated counterparts. The tables in Yanni and Dazzo (2010) show the results of eight experiments (#s 1, 2, 6, 10, 11, 12, 13, and 14) in which significant increases in the harvest index resulted from inoculation while increasing the N dose significantly and typically decreased the harvest index, indicating that unlike for inoculation, N-fertilization increased vegetative straw production more so than increased grain production. In the remaining 16 experiments, no significant changes in harvest index were observed due to inoculation or increasing the N application dose, indicating that the recommended N dose used in those experiments was within (did not exceed) the acceptable range that balanced vegetative and reproductive growth.

The results here indicate that this parameter is mostly a plant genetic property that is only slightly affected by agronomic factors like fertilization, inoculation, field location, soil texture, salinity, etc. In this translational research/technology study, inoculation and the N-fertilization doses, in most cases, worked together synergistically in providing the plant's demand for N and other nutrient requirements without seriously decreasing the harvest index values. This beneficial result is consistent with our previous work (Yanni et al., 1997, 2001) showing that rhizobial inoculation and N-fertilization contributed to rice vegetative growth and grain yield in parallel.

111.2.4 Inoculation and N Economy in Rice Agroecosystems

Increases in the N-fertilizer use efficiency (kilogram grain yield/kilogram N-fertilizer) translate to higher production of grain yield with less dependence on additional amounts of N-fertilizer inputs. It does not represent the absolute values of the N-fertilizer use efficiency, which requires ^{15}N analyses to measure how much of the plant nitrogen was derived from the applied N-fertilizer.

As anticipated, the "agronomic" N-fertilizer use efficiency decreased sharply with increased applications of N-fertilizer for both the inoculated and noninoculated treatments and for all the rice varieties under the different soil salinities encountered, with significantly higher mean differences for the inoculated versus the noninoculated treatments (Table 111.5). Its values were significantly higher for inoculated treatments in 18 (75%) of the 24 field experiments (Yanni et. al., 2010). By extrapolation, the rice grain yield of field plots inoculated with rhizobia plus intermediate doses of N-fertilizer was close or equal to that obtained with application of the full N-fertilizer dose without inoculation. This very significant experimental result clearly indicates that rhizobial inoculation can significantly improve rice grain production by counterbalancing the trade-off in reduced agronomic use efficiency of N-fertilizer with increasing the fertilizer doses, producing the desired result that rhizobial inoculation can reduce the need for additional N-fertilizer for production of higher grain yield or obtain the same grain yield from less field area.

Unlike the well-known suppression of symbiotic nitrogen fixation resulting from application of N-fertilizers in fields of nodulated legumes, the results here indicate that the degree of benefit to rice by inoculation with rhizobia was enhanced rather than suppressed by N-fertilizer application. That result means also that inoculation with rice

Table 111.5 The agronomic fertilizer N-use efficiency* in the experimental fields and adjacent farmer's fields as affected by rice varieties, soil salinity, inoculation, and N-fertilization doses

Rice Var.	N (kg/ha)	Noninoculated Control	Inoculated	N-Fertilizer Use in Farmer's Fields	Salinity Level (ppm)	N (kg/ha)	Noninoculated Control	Inoculated	N-Fertilizer Use in Farmer's Fields
Jasmine	48	152	180		A (0–2500)	48	191	203	65
	96	81	92	50		96	104	112	
	144	61	65			144	74	78	
Giza-177	48	150	173		B (2501–5000)	48	204	222	69
	96	86	97	66		96	103	113	
	144	62	70			144	74	78	
Giza-178	48	192	204		C (5001–7500)	48	191	205	50
	96	104	112	65		96	103	108	
	144	74	79			144	70	73	
Sakha-101	48	174	204		D (>7501)	48	200	216	58
	96	103	111	66		96	107	112	
	144	72	75			144	75	79	
Sakha-104	48	199	213	66					
	96	106	113						
	144	74	78						

*The agronomic fertilizer N-use efficiency: kg paddy yield/kg N-fertilizer applied.

rhizobial endophytes increased grain yield even when N was no longer the major limiting factor for grain production, clearly indicating that inoculation transforms the plant to make better use of the available N and other plant nutrient resources in the root zone that are not readily accessible by the plant without inoculation. The outcome of this experimentation program supports our previous studies showing benefits of inoculation in expanding the plant root architecture (biovolume, biomass and cumulative root length, and surface area), increased absorption and acquisition of nutrients by the plant, enhanced production of plant growth regulators, efficient use of insoluble inorganic phosphates in the rhizosphere area and organic phosphates inside the root system (Yanni et al., 1997, 2001), and probably other mechanisms by which this growth promotion was obtained.

111.2.5 Different Performance of Single- and Multistrain Inocula Preparations

A detailed comparison of each of the 24 experiments (Yanni and Dazzo, 2010) indicates that inoculants of certain rhizobial consortia perform better than with only a single rhizobial strain. Inoculation scored a mean increase in grain production of 0.706 ton/ha with multistrain inocula preparations versus 0.635 ton/ha with single-strain inocula, that is, 11.2% more grain yield when certain multistrain consortia were used.

111.2.6 Experimental Rice Productivity as Compared to National Figures of Rice Production in Egypt during 25 Annual Growing Seasons

Another benefit of rhizobial inoculation found in our studies was its ability to increase the potential of paddy yield production for certain rice varieties that exceed the maximum Egypt national rice productivity of 10.071 ton/ha scored in season 2006 (over 25 rice growing seasons extended from 1984 through 2011 and around 21.008 million h cultivated with rice during this quarter century period) when cultivated using the recommended amounts of N-fertilizers without inoculation (http://www.arc.sci.eg/BasicDataReport.aspx?OID=63&lang=en). Concerning mean yields of the rice varieties in the 24-experiment program (Table 111.2), the mean percentage increases were 11.5, 6.9, and 10.2 % for the rice varieties Giza-178, Sakha-101, and Sakha-104, respectively. The increased rice production capacity occurred in 20 of the 24 inoculation experiments when it could not be achieved by N-fertilizer application alone. Inoculation with rhizobia promoted grain yield to levels that exceeded that national production figure in 7 experiments with application of 48 kg N-fertilizer/ha, increased to 15 experiments with 96 kg N-fertilizer/ha, and in 21 experiments with the full recommended application rate of 144 kg N/ha. However, this variation cannot be attributed solely to contributions to grain yields related to the biofertilization practice, as the national production figures were calculated using results

obtained from a wide range of rice cultivation areas with a minimum of 453.6 thousand ha cultivated in season 1991 to a maximum of 743.0 thousand ha cultivated in 2008, under uncountable agronomic variables involved.

111.2.7 Comparison of Rhizobia with Other Biofertilizer Candidates for Rice

Previous field inoculation studies on rice have used N_2-fixing cyanobacteria, *Azospirillum* (see Chapter 90) and *Azotobacter* as biofertilizer inoculants (Subrahmanyan et al., 1965; Venkataraman 1966; Arora 1969; Jack and Roger, 1977; Rajarmamohan et al., 1978; Roger and Kulasooriya, 1980; Gupta et al., 1989; Yanni and Abdallah, 1990; Yanni and Osman, 1990; Yanni and Hegazy, 1990; Yanni 1991, 1992a, 1992b, 1996; Omar et al., 1993; Shahaby et al., 1993; Yanni and Abd El-Rahman, 1993; Yanni et al., 1996). Like with rhizobia, N-fertilizer supplements are required to obtain maximum grain yields even when rice is inoculated with the above mentioned diazotrophs, individually or in consortia containing two or all three of them (Yanni and Abd El-Fattah, 1999 and references therein). However, the performance of rhizobial inoculants differs from those other plant growth-promoting rhizobacteria in two major ways. First, the appropriate rhizobia can benefit both the legume and the cereal sharing the same crop rotation, a phenomenon quite exemplified and easy concluded from results in this research program, whereas the other inoculants are only used for the cereal crops. In Egypt, ~ 67% of rice is cultivated as a summer crop in rotation with berseem clover. The other winter legume crops used in rotation with rice include legumes like faba bean, lentil, bean, lupines, fenugreek, and alfalfa that may also be rotated with rice as a summer crop in the same fields, so that rotation can help to maintain and perpetuate the populations of clover rhizobia involved in both clover–microbe and rice–microbe interactions. Second, the benefits of the rhizobial inoculants to rice occur at each stage of its development, beginning with seed germination and extending through grain maturity (Dazzo et al., 1999; Biswas et al., 2000a; Biswas et al., 2000b; Yanni et al., 2001, 1997; Dazzo and Yanni, 2006), whereas the benefits to rice provided by the other biofertilizer inoculants mentioned earlier are expressed only during its maximum "N-limitation stress period" when an external source of N is required at the tillering stage that extends from 15 to ~40 days post rice seedling transplantation (Yanni and Abd El-Fattah, 1999 and references therein).

111.2.8 Biosafety: A Pertinent Issue in the Biofertilization Technology

The importance of this issue is illustrated in some of our earlier gnotobiotic culture studies (Yanni et al., 1997, 2001). We found that some natural rice endophyte strains of clover rhizobia were active root nodulators and efficient N fixers on berseem clover, some others were inhibitory to rice growth, and a few of those natural rice endophyte strains of rhizobia in our collection were efficient PGP+ on rice but *lethal* pathogens on berseem clover! Since all these beneficial, neutral, and harmful types of endophytic rhizobia–rice interactions exist in nature, it is important to screen for and identify possible detrimental plant–bacteria symbiotic or associative combinations beforehand to avoid catastrophic inoculation outcomes to either crop under field conditions. We made sure beforehand that each of the seven strains of rhizobia that were included in our experimental field program was pretested under gnotobiotic conditions and found to be Nod+ and Fix+ on berseem clover and PGPR+ on many rice varieties (Yanni et al., 2001). Strains belonging to three of the 10 strain genotypes were excluded from field experiments based on these (in)compatibility screen tests in the lab.

111.2.9 Justification of Conclusive Recommendations Derived from the Studies

The translational research/technology program exemplified here can serve as a successful example of how deployment of well-selected biofertilizers can represent an integral component of sustainable nutrient management for rice production and can help farmers grow better, more productive rice crops in an environmentally safe way when soil fertility (especially available N) is a limiting factor. Thus, in addition to its well-known use as a micropartner in the N_2-fixing root-nodule symbiosis with legumes, rhizobia can be used as a microbial biofertilizer that can significantly enhance rice production through a different pathway of rice–rhizobia endophytic association. The benefit provided by the rhizobial biofertilizer is enhanced rather than suppressed with N-fertilizer supplementation, consistent with a mechanism that involve bacterial induction of an increased ability of the plant to sequester and utilize N from the available soil N pool rather than from de novo, *in planta* biological nitrogen fixation. They are not intended to replace the recommended rice farming practices for N-fertilizer management (amounts, N-fertilizer source, and timing of application), which were defined through a significant amount of multidisciplinary teamworks involving soil, water management, and plant nutrition scientists (http://www.arc.sci.eg/InstsLabs/Default.aspx?OrgID=1&lang=en), agronomists (http://www.arc.sci.eg/ResearchStations.aspx?&lang=en#S307), plant pathologists (http://www.arc.sci.eg/InstsLabs/Default.aspx?OrgID=6&lang=en), climate specialists (http://www.arc.sci.eg/InstsLabs/Default.aspx?OrgID=23&lang=en), entomologists (http://www.arc.sci.eg/InstsLabs/Default.aspx?OrgID

=5&lang=en), field experimentation and agroeconomy specialists (http://www.arc.sci.eg/InstsLabs/Default.aspx?OrgID=9&lang=en), agriculture engineering specialists (http://www.arc.sci.eg/InstsLabs/Default.aspx?OrgID=12&lang=en), and extension experts (http://www.arc.sci.eg/InstsLabs/Default.aspx?OrgID=10&lang=en). Instead, it complements those recommendations by providing an additional tool to enhance better plant growth and performance and N-fertilizer use efficiency in an environmentally friendly way that increases crop productivity without excessive use of additional N-fertilizer applications. However, cases where inoculation increased grain production despite no statistically significant increase obtained in response to the maximum recommended dose of N-fertilizer (Yanni and Dazzo 2010) indicate that the benefits of rhizobial inoculation on grain production extend beyond its alleviation of N-limitation.

These translational studies indicate that use of these environmentally friendly biofertilizer inoculants can now be recommended with sufficient supporting data to improve agriculture economy and sustainable agroecosystem maintenance of rice grain production where the benefits of such biotechnology are most urgently needed. However, because rice production often remains "N responsive" even when combined with rhizobial inoculation, further efforts must be exerted to reach the ultimate goal of fully eliminating the need for N-fertilizer inputs to obtain reasonable economic rice grain production. Perhaps the solution to achieve that goal is a critically tested/formulated biofertilizer(s) containing a mixed inoculant consortium that includes our best-performing rhizobial strains plus highly selected free-living N fixers like cyanobacteria, "associative" diazotrophs like azospirilla, and other plant growth-promoting rhizobacteria like *Pseudomonas*. We have been exploring that proposed updated plan and have obtained some very promising outputs through another translational large-scale biofertilization program in further experimentation fields in and outside the Nile Delta. Reports of those studies are forthcoming.

111.3 TAKE-HOME LESSONS OF THIS RESEARCH PROGRAM

Assuming that the results summarized here accurately reflect the potential benefits of this new agricultural biotechnology based on exploitation of a natural resource (natural rhizobial endophytes of cereals), the following outcomes can be expected:

1. Increased cereal crop yields above what is reached using organic and inorganic fertilizers alone, with a reduction in the amount of currently recommended fertilizer input without biofertilization. Economically, this can assist farmers to increase their yield figures while reducing the dependence on additional N-fertilizer inputs to achieve higher yields from important rice varieties, including under a wide range of soil salinity stresses. The farmers then do not need to increase the used land area to obtain similar yields as those reached by biofertilization with the tested strains.
2. Improvements in human nutrition by the increased production of rice with its multiple nutritional values.
3. Decreased environmental pollution and health risks originating from excessive use of inorganic N-fertilizers. However, further studies are needed to verify the reduction in costs of disease treatments associated with excessive use of agrochemicals and the economic benefits from increasing individuals' work abilities.
4. Decreased energy needed for production, transportation, and distribution of fertilizers and directing it to other socioeconomic and industrial uses.
5. A better understanding of how farmers can practice sustainable agriculture by utilizing biofertilizers as a safe and effective alternative to synthetic fertilizers.
6. Promotion of cooperation between research institutions on one side and private sectors represented by farmers and agricultural biotechnology industries on the other.

One of the many lessons we have learned from this study was stated well by Leonardo da Vinci: "Look first to Nature for the best design before invention." Although it is also a huge challenge, the research presented here has shown that the exploitation of naturally selected and significantly screened beneficial plant–bacterial associations is the sound and environmentally friendly way that has high probability for success. This is particularly relevant for the design of strategies derived from the use of genetic engineering for sustainable agriculture. By implementing these words of wisdom, biotechnology can capitalize upon such understanding in efforts to enhance agricultural production in ways that are consistent with complex, evolved biological relationships ascending from the foundation of natural microbial populations associating with crop plants on Earth.

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Section 19

Accessory Chapters

Chapter 112

N Fixation in Insects: Its Potential Contribution to N Cycling in Ecosystems and Insect Biomass

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112.1 INTRODUCTION

Nitrogen plays a central role in most metabolic processes and therefore represents a critical element in the growth of all organisms (Mattson, 1980). However, whereas many prokaryotes can assimilate atmospheric N_2 and then are autotrophs for this nutrient, the higher organisms such as plants, animals, and fungi do not harbor this important physiological process. Although gaseous N_2 covers each centimeter of the land and N cycles through the ecosystems, organically bound nitrogen represents a limiting factor in many ecosystems and for many species. The main source of an additional supply of organic N to the ecosystems is the biological fixation of N from the atmosphere by a selected group of prokaryotes (i.e., Rhizobiales and green sulfur bacteria) (Young, 1992) which possess the complex enzymatic nitrogenase machinery (LeBauer and Treseder, 2008; see Chapter 2). These prokaryotes reduce N_2 and convert it into forms that can be utilized by animals, plants, and fungi (Nardi et al., 2002). The global biological nitrogen fixation amounts to ≈ 110 and 140 teragrams in terrestrial and oceanic ecosystems, respectively (Fowler et al., 2013; Gruber and Galloway, 2008; Nardi et al., 2002; Vitousek et al., 2013; see also Chapter 84).

Plants like the legumes establish nutritive mutualisms with prokaryotes (rhizobia), which allow them to have direct access to the *de novo* fixed N. By contrast, the survival, development, maintenance, and growth of arthropods are believed to depend on organic diets with their content of organically bound N, and N often limits their development (Douglas, 2009). When arthropods feed on a nonbalanced diet, they must obtain additional N from other food sources. Here, we present evidence in favor of the existence of N fixation by endosymbiotic prokaryotes that reside in insect midguts: insect–prokaryote mutualisms might be as important for the N balance in many terrestrial ecosystems as the well-known legume–rhizobia mutualism.

112.2 NITROGEN ACQUISITION BY INSECTS

Insects have rather uniform nutrient requirements but live on very diverse diets. Therefore, the development, behavior, and reproduction of herbivorous insects in particular critically depend on the quality of the plant parts on which they feed and the content in limiting nutrients in these diets (Awmack and Leather, 2002; Scriber and Slansky Jr, 1981). In particular, while animals consist mainly of proteins (≈50%) (Mattson, 1980), most diets of herbivorous insects (such as wood, leaves, fruits, phloem sap, or nectar and other plant exudates) are very low in nitrogen. The average N content of an insect is usually 10–20 times higher than the N content of the autotrophic organisms that represent its diet. An exception appears to be only those parts of plants that have particularly evolved to serve as food source for animal mutualists, such as certain fruits, elaiosomes, and the food

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bodies of specific ant plants (Heil et al., 2004; Heil et al., 1998; Orona-Tamayo et al., 2013). For this reason, even in some human cultures, insects are the most important food source (Barker et al., 1998; Fagan et al., 2002; Finke, 2005).

Likewise, many of those insects that feed on a mainly or strictly vegetarian diet must dramatically alter the relative composition of nutrients in their diet, upgrade some nutrients, and discriminate against others in order to reach the balanced composition in their alimentation (Douglas, 2009; Mayr et al., 2010). Low nitrogen content in the diet can cause severe problems with the physiology of an insect (Benemann, 1973; Wyatt et al., 1956) and, in fact, of all herbivorous animals. For example, the fecundity of the aphid (*Drepanosiphum platanoidis*) is limited directly by the availability of amino acids in the phloem (Dixon, 1970; Douglas, 1993). The Colorado potato beetle (*Leptinotarsa decemlineata*) can use sugars and fatty acids as energy source during flight, but this species also oxidizes the amino acid proline as the main fuel for flight (Arrese and Soulages, 2010; Brouwers and de Kort, 1979; Mordue and de Kort, 1978), as do many other insects such as honeybees, which depend on proline as the main energy source during the early phases of flight (Carter et al., 2006). Whereas adult bees collect nectar and pollen and convert it to honey that provides the energy for flight, pollen provides the N compounds for larval development (Camazine, 1993; Seeley, 1989). In other social insects, such as leaf-cutter ants (*Atta* or *Acromyrmex*), pieces of leaves are used to cultivate a fungus that is the food source for the colony (Craven et al., 1970; Haeder et al., 2009; Weber, 1966; Abril and Bucher, 2004; Bass and Cherrett, 1995). However, neither pollen nor fungal mycelia seem to contain sufficient protein to provide growing larvae with all their nitrogen needs.

This general imbalance between the biomass of herbivorous insects and the availability of their food plants has been expressed in "Tobin's ant-biomass paradox": according to normal quantitative ratios among different trophic levels, the nitrogen contained in what is considered food available to tropical arboreal ants seems not to be sufficient to maintain the ant biomass that exists in nature (Davidson et al., 2003; Tobin, 1995). The solution appears to be that many insects that live on low-N diets depend on associations with microbes that contribute to their nutrition. For example, termites and some wood-feeding beetles have been reported to fix N_2 from the atmosphere (Breznak et al., 1973; French et al., 1976; Kuranouchi et al., 2006; Morales-Jiménez et al., 2009; Morales-Jiménez et al., 2013). Because insects lack the enzymatic machinery for the reduction of N_2, the conclusion was that the insects gained this capacity via the establishment of an "endosymbiosis": a mutualism with resident N-fixing bacteria that live inside the host, allowing particular direct interactions between the partners (Kikuchi, 2009).

Cyanobacteria are the most widespread and major N fixers in freshwater or marine systems (Vitousek et al., 2002; see Chapter 84). These versatile organisms combine photosynthesis and N fixation in the same cell (Berman-Frank et al., 2001; Berman-Frank et al., 2003). In soil, microbes that biologically fix atmospheric N can either live freely in the substrate or interact with plant roots to establish nodular symbiotic relationships (Nardi et al., 2002; Sprent and Sprent, 1990; Vitousek et al., 2002). Surprisingly, evidence is now accumulating that the same prokaryotic taxa can also be found in insects, where they might fulfill the same function: to supply their host with organically bound N.

112.3 WHY ENDOSYMBIONTS RESIDE IN INSECTS?

Xylophagous insects such as termites and bark beetles represent a paramount example of insects with a low-N diet, because they feed on woody material that is rich in cellulose, hemicelluloses, and lignin but usually contains only ≈0.03–0.1% nitrogen (Breznak, 1982; Nardi et al., 2002; Prestwich et al., 1980). To enable protein synthesis and growth, these insects must have physiological adaptations to maintain their internal N pool by dietary supplementation or internal cycling of N (La Fage and Nutting, 1978; Prestwich et al., 1980; Wood, 1978). Interestingly, microbial endosymbionts contribute significantly to these essential processes.

In fact, many insects depend on gut endosymbiotic bacteria to balance their nutrition (Chandler et al., 2008; Eilmus and Heil, 2009; Nardi et al., 2002). For example, the evolution of herbivory in ants has been suggested to have been facilitated dramatically by the establishment of mutualistic interactions with gut endosymbiotic bacteria (Russell et al., 2009). Endosymbiotic bacteria in general aid with processes as diverse as the digestion of complex and difficult-to-digest types of food, detoxification, the synthesis of vitamins and amino acids, and the defense against pathogens (Brownlie and Johnson, 2009; de Souza et al., 2009; Douglas, 1998; Douglas, 2006; Engel and Moran, 2013; Ishak et al., 2011; Lai et al., 1996; Scott et al., 2008; Shi et al., 2013; Zientz et al., 2006; Zientz et al., 2004). The class Insecta is composed of more than 1,200,000 species, and approximately half of them are estimated to harbor endosymbiotic bacteria (Buchner, 1965; Douglas, 1989; Kikuchi, 2009). The most specialized of these bacteria are located in the cytosol of specialized cell structures, the so-called bacteriocytes (Moran and Telang, 1998; Swiatoniowska et al., 2013; Zientz et al., 2005).

Why have these interactions been established? In the end, numerous examples of free-living taxa demonstrate that to survive, N-fixing prokaryotes do not necessarily require any mutualism with a plant or animal. In this context, the predictability of the abiotic and biotic environment (temperature, salinity, humidity, pH, nutrient supply) that

the arthropod gut offers might be an important factor that favors endosymbiosis. As in the case of the legume–rhizobia mutualism, the symbionts can acquire energy for their reproduction in a very reliable way and are protected from most hazardous environmental threats (Nardi et al., 2002). The insects, by contrast, gain access to the biosynthetic diversity that is represented by the prokaryotes. Moreover, in the case of vertically transmitted endosymbionts, the direct interdependency of fitness of both partners helps to maintain mutualistic behavior (Gibson and Hunter, 2010).

112.4 BACTERIAL COMMUNITIES ASSOCIATED WITH INSECTS

Insect endosymbionts are extremely diverse and stem from different phyla that comprise the protists, fungi, archaea, and bacteria (Anderson et al., 2012; Engel and Moran, 2013). Their coevolution with insects has allowed these endosymbionts to colonize the digestive tracts of virtually all insects and in exchange to offer different functions to the host insect. The diet on which the host lives can clearly influence the persistence of endosymbionts, and the proportion of taxa is very different among arthropods (Engel and Moran, 2013). Whereas some taxa of endosymbionts are commonly reported and are universally found in the digestive tracts of insects, others represent specific bacterial residents of certain insect hosts (Anderson et al., 2012).

In some insects, microbial residents are considered as obligate, because they are required for specific vital events in the host; often are transmitted vertically; and then usually live as intracellular symbionts. Examples for this highly special class of endosymbionts are *Buchnera* and *Wigglesworthia* (Akman et al., 2002; Brownlie and Johnson, 2009; Koga et al., 2003). The second group of commonly reported endosymbionts comprises intestinal bacteria that commonly live in the gut lumen, that is, within the insect but outside its cells. These extracellular endosymbionts are considered as facultative symbionts and are usually not required for vital events (Moran et al., 2008). However, the composition of symbionts differs even among closely related species of arthropods, which indicates a certain degree of specificity in these interactions (Fukatsu and Nikoh, 2000; von Dohlen et al., 2001).

Phylogenetic analyses based on the sequencing of the 16S ribosomal DNA indicate that the majority of insect endosymbionts are β- and γ-proteobacteria (Brauman et al., 2001; Breznak, 2000; Degnan et al., 2004; Dillon and Dillon, 2004; Dohlen et al., 2013; Gruwell et al., 2012; Morales-Jiménez et al., 2009; von Dohlen et al., 2001; Wernegreen, 2002; Wernegreen et al., 2009). Social insects such as ants have motivated particular scientific scrutiny due to their abundance in tropical regions (Davidson et al., 2003; Hölldobler and Wilson, 1990; Wilson and Hölldobler, 2005), and the presence of endosymbionts was first reported from *Formica* and *Camponotus* ants (Baumann et al., 2006). Later studies on other ant species found a number of specialized gut bacteria, among which β- and γ-proteobacteria and Rhizobiales were dominating, and significantly increased the number of microbial taxa that are known to be associated with ants (Eilmus and Heil, 2009; Lee et al., 2008; Martinez-Rodriguez et al., 2013; Rey et al., 2013; Stoll et al., 2007). Interestingly, the taxonomic composition of the typical bacterial communities in ants was very similar to those of termites, although ants in general possess bacteria at lower abundance (Engel and Moran, 2013).

Other arthropods for which intestinal bacteria are frequently being reported are phloem sap-feeding insects such as aphids, whiteflies, psyllids, and mealybugs (Baumann et al., 2006). The shared nutritional features of their diet comprise high content of carbohydrates and very low contents of amino acids and other N-based compounds (Sandström and Moran, 1999). Therefore, these insects are loaded with different endosymbionts in their midguts, most commonly from the β- and γ-proteobacteria, although the specific taxa differ from those reported for ants (Baumann et al., 2006; Gosalbes et al., 2010). Moreover, *Buchnera aphidicola*, *Candidatus* (*Ca.*) *Carsonella ruddii*, *Ca. Portiera*, and *Ca. Tremblaya* are frequently reported to be present in phloem-feeding insects (Clark et al., 1992; Gosalbes et al., 2010; Jiang et al., 2013; Munson et al., 1991; Tamames et al., 2007; Thao et al., 2000). In fact, *B. aphidicola* and its aphid host are the best-studied example of an insect–endosymbiont association (Munson et al., 1991; Sasaki and Ishikawa, 1995; Shigenobu et al., 2000). Here, the endosymbiont is involved in the biosynthesis of essential amino acids, a function that has also been suggested for the prokaryotes residing in other insects that feed on phloem (Gosalbes et al., 2010; Tamames et al., 2007).

That gut endosymbionts are selected in adaption to the nutrition of their host can also be exemplified observing the case of termites. The midgut of termites is charged with cellulases that apparently are of fungal origin, and these are involved in the digestion of lignocelluloses and, thus, in the acquisition of carbon from woody material (Hyodo et al., 2000; Martin and Martin, 1979). However, other endosymbionts in termites have been reported to support the fixation or recycling of nitrogen (Ohkuma et al., 1999; Potrikus and Breznak, 1980c).

Like termites, bark beetles live on an N-poor diet, and their endosymbiotic fauna indeed is similar to that found in termites (Adams et al., 2010; Bois et al., 1999). For example, the turpentine bark beetle (*Dendroctonus valens*) is associated with a diverse bacterial community. Interestingly, some well-characterized endosymbionts in these beetles are genera such as *Cellulomonas* and *Cellulosimicrobium*, which show cellulase activity in the beetle midgut (Morales-Jiménez et al., 2009). Furthermore, nitrogen fixation was found both

in larvae and adult beetles, although the first enterobacteria that were isolated from these beetles yielded negative results concerning N fixation *in vitro* (Morales-Jiménez et al., 2009). However, a diazotrophic bacterium [*Raoultella terrigena*] was isolated from *Dendroctonus rhizophagus* and *D. valens* recently and was found to be involved in N fixation and recycling (Morales-Jiménez et al., 2013). Even the aforementioned (Section 112.2) insects commonly consume solutions that are rich in carbohydrates but comparably poor in N, such as extrafloral nectar (Heil et al., 2005; Hölldobler and Wilson, 1990; Janzen, 1974), plant wound secretions, and insect honeydew (Delabie, 2001; Douglas, 2006). Consequently, several studies discovered bacteria in ants whose phylogenetic affiliation indicated their possible involvement in the supplementation of essential nutrients via N fixing and N recycling (Breznak et al., 1973; Eilmus and Heil, 2009; Feldhaar et al., 2007; Potrikus and Breznak, 1981).

112.5 NITROGEN FIXATION AND RECYCLING AS SUPPORT FOR INSECT NUTRITION

One main function of endosymbionts in host insects appears to be the upgrading of the N composition of their diet, although these bacteria also supplement unbalanced diets with essential amino acids and other vitally important compounds such as vitamins (Fan and Wernegreen, 2013). With the introduction of the acetylene reduction (AR) assay, researchers gained a sensitive, rapid, and economic method to estimate the N fixation by nitrogenase activity (Hardy et al., 1973; Stewart et al., 1967). The first class of insects for which this assay was employed was termites. Breznak et al. (1973) and Benemann (1973) applied the AR assay to intact termites and animals from which the guts had been removed and found that N fixation depended on the presence of the gut content. Consequently, they speculated that gut endosymbionts are involved in this process. Then, other wood-feeding insects were evaluated with similar results, for example, bark beetles (Bridges, 1981), cockroaches (Breznak et al., 1974), and soil beetles (Citernesi et al., 1977). Based on the positive results of these screenings, the researchers concluded that endosymbionts are involved in the nitrogen metabolism of the insect.

The development of further molecular tools – and in particular the detection of the nitrogenase gene *nifH* – allowed for more detailed studies (Kneip et al., 2007). For example, various endosymbionts that were isolated from *D. valens* (a bark beetle in pine) were found to fulfill different metabolic functions such as the detoxification of secondary plant compounds and the fixation of N (Morales-Jiménez et al., 2009; Morales-Jiménez et al., 2013). Similarly, certain ant species such as *Tetraponera* are loaded with bacteria that were identified as *Burkholderia*, *Flavobacteria*, and Rhizobiales, that is, in part with taxa known from root nodules of legumes and known to fix nitrogen (Lee et al., 2008), and also, the endosymbiont community of *Pseudomyrmex ferrugineus*, an obligate ant plant inhabiting *Acacia* myrmecophytes, contained multiple taxa that are known as N fixers (Eilmus and Heil, 2009).

The acquisition of nitrogen from food and the supplementation via the assimilation of atmospheric N could be a primary function of endosymbionts in the insects. However, insects lose, by excretion, *ca*. 55–80% of their nitrogen (Bursell, 1970) in the form of uric acid (Nation and Patton, 1961) or urea (Feldhaar et al., 2007) or of primary degradation products such as allantoin (Gaines et al., 2004), allantoic acid (Wadano and Miura, 1976), and ammonia (Scaraffia et al., 2005). Therefore, obtaining nitrogen from these wastes can provide a further rich source of this limited resource in insects (Sabree et al., 2009). Indeed, several insects are capable of storing nitrogen in various inorganic forms and have developed relationships with microbes that can make this N reaccessible to the insect host (Cochran, 1985; Morales-Jiménez et al., 2013; Weihrauch et al., 2012). In *Periplaneta americana*, an omnivorous cockroach, uric acid is stored in the fat body, which serves as metabolic reserve and can release the nitrogen when the insect is deprived of food protein (Cochran, 1985; Sabree et al., 2009). Interestingly, the fat body of this insect harbors *Blattabacterium* (López-Sánchez et al., 2009), which can recycle ammonia and urea although it is unable to assimilate uric acid, because it lacks genes encoding the uricases that are involved in uric acid catabolism (Sabree et al., 2009). In this case, the uricases may be expressed by the insect host or by other endobacteria, which can metabolize uric acid to form the ammonium that *Blattabacterium* then uses to synthesize amino acids (López-Sánchez et al., 2009; Sabree et al., 2009). Apart from cockroaches, *Blattabacterium* is found also in primitive termites such as *Mastotermes darwiniensis*, where it contributes a similar function to the insect host (Bandi et al., 1995). Termites are known to store uric acid in the fat body (Potrikus and Breznak, 1981), but they lack uricase activity and endosymbiont bacteria in their fat body (Potrikus and Breznak, 1980a). However, the uric acid can be transported to the insect gut where it is converted by uricolytic microorganisms (Sabree et al., 2009). For example, in termites such as *Reticulitermes* sp., uric acid is transported from the hemolymph to the hindgut, where *Bacteroides* and *Streptococcus* strains are responsible to convert uric acid to ammonia (Potrikus and Breznak, 1980a; Potrikus and Breznak, 1980b, 1980c; Potrikus and Breznak, 1981). The shield bug, *Parastrachia japonensis*, produces uric acid and other waste nitrogenous compounds such as allantoin, allantoic acid, and urea. Here, the microbial endosymbiont *Erwinia*, which was detected in the midgut, synthesizes uricolytic enzymes such as uricase, allantoinase, and allantoicase, which convert these waste products to

ammonium. Ammonium, in turn, serves as substrate for other bacteria that synthesize and finally excrete amino acids to the insect gut (Kashima et al., 2006). Also, other insects such as *D. valens* or *D. rhizophagus* beetles or the planthopper (*Nilaparvata lugens*) contain gut microbes involved in the conversion of uric acid to maintain the N requirements of the insects (Hongoh and Ishikawa, 1997; Morales-Jiménez et al., 2009; Morales-Jiménez et al., 2013), as it has also been described for *Camponotus* ants, which harbor intracellular *Blochmannia* strains. This endosymbiont contains a complete urease cluster and thus should be able to hydrolyze urea into CO_2 and ammonium, the latter of which then can serve as substrate to produce amino acids in the ant host (Feldhaar et al., 2007). Therefore, several *Camponotus* species are attracted to natural aqueous solution of urea (bird feces or human urine] (Feldhaar et al., 2007; Shetty, 1982). Finally, the endosymbionts in many social insects can upgrade the quality of the diet of their host by utilizing nonessential constituents and transforming them into compounds that are essential for the host (Cook and Davidson, 2006; Feldhaar et al., 2010).

112.6 THE IMPORTANCE OF N FIXATION BY INSECTS IN ECOSYSTEMS

Nitrogen is a limited compound in all ecosystems. Arthropods that harbor nitrogen-fixing endosymbionts might represent an as yet underestimated source of additional N (Nardi et al., 2002). Considering their great abundance and ecological importance, the ability of termites to fix atmospheric N might play a particularly important role in the fertilization of ecosystems with organic nitrogen (Ohkuma et al., 1999). Estimated mean rates of N fixation by an intact termite colony in rain forest are about 0.12 kg/ha/day (Breznak, 1984), which would amount to 43.8 kg/kg//ha/year. For insects such as beetles and millipedes, their N fixation was estimated to be around 0.02 kg/ha//year (Hackstein and Stumm, 1994). In summary, the fixation of N in the guts of arthropods was estimated to represent 10–40 kg/ha/year and, thus, might indeed represent a significant contribution to the N balance of certain ecosystems (Nardi et al., 2002). However, much more research will be required before this mechanism can be considered a quantitatively important source of N.

112.7 CONCLUSIONS

Nitrogen fixation is restricted to specific prokaryotes that contain nitrogenase, an enzyme complex that catalyzes the reduction of atmospheric N_2 to ammonia. However, multiple insects have established interactions with endosymbiotic prokaryotes. In these interactions, the insect host benefits from the metabolic capabilities of certain bacteria to fix N and obtains N-containing organic compounds from the endobacteria. In return, the bacteria obtain a specific and stable habitat for their protection, including reliable supply of the required nutrients for their reproduction. Besides the fixation of atmospheric N_2, the recycling of N represents a further mechanism by which endosymbiotic bacteria in arthropods contribute to the optimization of the N balance of their hosts. The insects release different N waste products that bacteria use as substrates for the biosynthesis of essential amino acids, which fulfill the nutritional requirements of the host. The fixation or recycling of N by endosymbionts of insects might represent an important and as yet underestimated source of organic N in many ecosystems. However, the successful fixation of N depends on multiple environmental factors and has seldom been quantitatively demonstrated *in vivo* for the prokaryotes that reside in insects. Determining the significance of N fixation by insect endosymbionts will require much more quantitative work that includes multiple taxa of insects and studies of them in their natural environment.

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Chapter 113

Rapid Identification of Nodule Bacteria with MALDI-TOF Mass Spectrometry

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113.1 INTRODUCTION

The Leguminosae family includes more than 19,000 species of plants (Lewis et al., 2005), the majority of which may form nitrogen-fixing associations with diverse soil bacteria generically known as rhizobia (Masson-Boivin et al., 2009). Efficient reduction of atmospheric nitrogen (N_2) by the symbiotic forms of rhizobia known as bacteroids occurs mostly inside plant cells of specialized root or more rarely stem organs called nodules. Within each of these N_2-fixing nodules, rhizobia establish long-lasting intracellular colonies of up to thousands of bacteroids per infected plant cell (Batut et al., 2004). To direct free-living rhizobia to nodule cells, most legume crops form transcellular infection threads (IT) that initiate at the tip of receptive root hairs, cross several layers of the root epidermis and cortex, and ultimately reach the newly established nodule primordia (Gage, 2004). As a single bacterium entrapped in a curled root hair is sufficient to initiate IT formation, most N_2-fixing nodules harbor clonal-like bacteroid populations. Throughout the infection process, host plants and rhizobia exchange a number of molecular signals that coordinate IT development and nodule morphogenesis as well as prevent [or minimize] infection by pathogenic bacteria or ineffective strains (Gibson et al., 2008; Perret et al., 2000; Ryu et al., 2012). In spite of the apparent complexity of the infection processes that lead to functional N_2-fixing nodules and the selective screening for proficient bacteroids implemented by most legume hosts, rhizobia form a surprisingly heterogeneous collection of bacteria (Gyaneshwar et al., 2011; Masson-Boivin et al., 2009). Lateral transfer between unrelated soil bacteria of the genes required for nodulation and nitrogen fixation has been highlighted as one of the reasons for such diversity of microbial solutions to the legumes' need for symbiotically reduced forms of nitrogen (Rogel et al., 2011).

Developments in molecular techniques and a renewed interest in probing native legumes collected throughout the world for novel symbiotic strains have led to the identification of "unusual" rhizobia, such as members of the beta subdivision of proteobacteria (now known as ß-rhizobia) (Chen et al., 2001; see Chapters 17, 89) or bradyrhizobia that do not produce nodulation factors (Giraud et al., 2007; see Chapter 28). DNA–DNA hybridization techniques

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and sequence analyses of 16S rRNA genes have been the predominant molecular tools for bacterial taxonomic studies. Both techniques show intrinsic limitations, however. DNA–DNA hybridization results have been reported to vary between laboratories and were labor-intensive to acquire (Rosselló-Mora, 2006), while 16S rRNA-based analyses (see Chapter 3) have been found to be inadequate for distinguishing closely related species/strains or describing the complex evolution of rhizobial genomes that are prone to genetic recombination and horizontal gene transfer (Broughton, 2003; Freiberg et al., 1997; Sullivan and Ronson, 1998; van Berkum et al., 2003). Multilocus sequence analysis (MLSA) of faster-evolving protein-encoding genes has thus been proposed as an alternative genotyping method that provides adequate delineation of species, including those within distantly related genera such as *Bradyrhizobium* or *Sinorhizobium* (Martens et al., 2008; Nzoué et al., 2009). Often used as markers in MLSA-based population studies (van Berkum et al., 2006, Aserse et al., 2011), the selection of *dnaK*, *glnA*, *gyrB*, *recA*, and/or *rpoB* as reference housekeeping genes (see Chapter 3) has been questioned recently following a comparative analysis of more than 20 complete rhizobial genomes (Tian et al., 2012; Zhang et al., 2012). Regardless of the genetic markers selected for MLSA studies or the molecular tools chosen for describing the unknown nodule isolates, all DNA-based methods of identification require the isolation and amplification of pure bacterial cultures *in vitro*, followed by preparations of genomic DNA samples for each of these strains.

In addition, the isolation of symbiotic strains from soil samples or plant nodules collected in fields can be a tedious process. Like other root surfaces, nodule epithelia are colonized by diverse rhizospheric bacteria, some of which can resist standard surface sterilization procedures (e.g., spore-forming microbes) or find protection against sterilizing agents within the nodule epidermis or cortex. Once plated on growth media, these contaminants often outgrow symbiotic strains, thus necessitating further time-consuming purification steps to enrich and ultimately cultivate isolated colonies of true rhizobia. As some bradyrhizobia have been reported to be particularly slow growers (Xu et al., 1995), weeks if not months can be required to obtain a definitive strain identification from the time nodules are harvested or soil samples collected. These labor-intensive isolation procedures combined with time-consuming identification protocols prompted us to assess the potential of mass spectrometry (MS) in typing nodule bacteria.

In many diagnostic laboratories, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS has replaced the more costly and laborious DNA-based procedures for the identification of clinical isolates (Seng et al., 2009). In procedures involving intact cell MS, small samples taken from single colonies are deposited on a target plate and mixed with a crystalline matrix (e.g., sinapinic acid) that facilitates the ionization of cellular components. When exposed to a laser beam, the matrix-embedded cells are vaporized and the cellular components released and ionized. The resulting charged molecules are accelerated in an electric field and targeted toward a detector that measures their time of flight: lighter molecules flying faster than heavier ones. The vast majority of molecules documented in this way are single-charged cellular proteins that are recorded as peaks with distinct mass/charge (m/z) values. Together, these recorded peaks form complex spectra or fingerprints that have been shown to be specific to the groups of cells deposited on the target plates. For strain identification, each MALDI-TOF MS spectrum is ultimately matched against a database of reference spectra that has been established for bacteria, yeasts, or fungi grown in standardized conditions [for a review, see Bader (2013)]. Archived reference spectra [also called SuperSpectra^{TM} in the Spectral Archive and Microbial Identification System (SARAMIS^{TM}) database] include lists of selected m/z biomarkers that are family, genus, or species specific. Accurate identification of clinical or environmental isolates depends upon the robustness of the database and the choice of strains used to compute the reference spectra (Benagli et al., 2012). When compared against other molecular methods routinely used for the identification of pathogenic strains in the field of medical diagnostics, MALDI-TOF MS has been found to be faster, cost-effective, and less prone to false positives (Cherkaoui et al., 2010). In addition, a number of studies have shown that clustering analyses of MALDI-TOF MS spectra are often congruent with phylogenies based on 16S rRNA, *rpoB*, *gyrB*, and/or *nifH* genes (Benagli et al., 2012; Hahn et al., 2011).

113.2 FROM DNA- TO PROTEIN-BASED MOLECULAR SIGNATURES FOR RHIZOBIA

Initially, the possibility to reliably identify symbiotic rhizobia via MALDI-TOF MS was examined using 50 selected strains belonging to the *Ensifer* and *Rhizobium* genera (Ferreira et al., 2011). Although this study confirmed that fast-growing rhizobia cultivated on media plates could be correctly and reliably identified by MALDI-TOF MS, two important limitations remained: (i) the procedure still needed well-purified strains as starting materials, and (ii) commercially available databases of reference spectra were mostly designed for typing pathogenic bacteria and do not include type strains for all of the known rhizobia genera. Taking advantage of the natural amplification of endosymbiotic rhizobia that occurs inside plant nodules, we have shown that MALDI-TOF MS-based identification of bacteroids found inside crude nodule extracts is both accurate and sufficiently discriminative to distinguish strains belonging to the same

Sinorhizobium fredii species (Ziegler et al., 2012). Thus, MALDI-TOF MS-based identification of nodule bacteria effectively bypasses the time-consuming and labor-intensive steps of purifying nodule isolates followed by preparations of genomic DNA. Incremental results on these premises are discussed in the following sections.

113.3 METHODS

113.3.1 Microbiological Techniques

The identity of all strains of rhizobia used in this work was confirmed by 16S rDNA sequencing. Unless specified otherwise, bacterial cells were grown for several days at 28°C in/on TY or YM media, using antibiotics at final concentrations of 50 µg ml^{-1}.

113.3.2 Preparation of Bacterial Samples for MALDI-TOF MS Analyses

To isolate bacteroids, nodules (either fresh or frozen) were crushed into 400 µl of sterile ddH_2O, and the resulting homogenates free of nodule debris were centrifuged at 20,000 *g* to collect bacteroids. To remove plant leghemoglobins, bacterial pellets were washed three times with 200 µl of sterile ddH_2O prior to spotting MALDI steel target plates. Free-living rhizobia were spotted directly from media plates. Each bacterial spot was then overlaid with 1 µl of matrix consisting of a saturated solution of alpha-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, Buchs, Switzerland) in 33% acetonitrile (Sigma-Aldrich) and 33% ethanol, supplemented with 3% trifluoroacetic acid, and air-dried for a few minutes at room temperature.

113.3.3 MALDI-TOF MS Fingerprinting

Rhizobial identification was carried out on a MALDI-TOF MS Axima™ Confidence machine [Shimadzu-Biotech Corp., Kyoto, Japan], with a detection in the linear positive mode at a laser frequency of 50 Hz and a mass range from 3,000 to 20,000 Da. Acceleration voltage was 20 kV, and the extraction delay time was 200 ns. A minimum of 10 laser shots per sample was used to generate each ion spectrum. For each sample, a total of 50 protein mass fingerprint profiles were averaged and processed using the Launchpad™ 2.8 software [Shimadzu-Biotech Corp., Kyoto, Japan]. Raw spectra were processed with Launchpad™ 2.8 using as settings the advanced scenario from the Parent Peak Cleanup menu, a peak width of 80 chans, a smoothing filter width set at 50 chans, a baseline filter width of 500 chans, and a threshold apex method set to dynamic. The threshold offset was set at 0.020 mV, with a threshold response factor of 1.2. Each target plate was first externally calibrated using spectra of the reference strain *Escherichia coli* DH5a.

113.3.4 Cluster Analyses

MALDI-TOF MS spectra were analyzed with SARAMIS™ [AnagnosTec, Potsdam-Golm, Germany]. For cluster analysis of protein mass fingerprints, binary matrixes were generated using the SARAMIS™ SuperSpectra tool and exported as text files. Datasets were imported into the PAST software [Natural History Museum, University of Oslo, Norway], and multivariate cluster analysis was performed using the Simpson algorithm in order to calculate distance matrixes. For the generation of dendrogram illustrations, distance matrixes formatted as nexus files were imported and processed using FigTree v1.3.1 software (http://tree.bio.ed.ac.uk/software/figtree/).

113.4 RESULTS AND DISCUSSION

One of the initial questions in using MALDI-TOF MS as a tool for typing rhizobia was whether spectra of free-living and endosymbiotic bacteria differed so much as to prevent their accurate identification. The answer was difficult to predict since metabolism and in some instances DNA content of bacteroids and free-living rhizobia differ considerably.

113.4.1 Typing Bacteroids versus Free-Living Rhizobia

Although nodules vary greatly in size and shape within the Leguminosae, most crops form root nitrogen-fixing nodules of either determinate or indeterminate types (Masson-Boivin et al., 2009; Sprent and James, 2007). Determinate nodules lack a persistent meristem, are spherical in shape, and contain mostly homogeneous populations of rhizobia found in developmentally synchronized infected plant cells. After a few weeks of symbiotic activity, these determinate nodules senesce and are replaced by new nodules formed elsewhere on younger root sections. In contrast, a nodule of indeterminate type has a longer lifespan, mostly because a persistent apical meristem provides a continuous source of new plant cells that become infected by bacteria. Once mature, indeterminate nodules are elongated and characterized by a gradient of developmental stages in which senescent plant and bacterial cells are replaced by those that divide in the apical meristem.

Regardless of the nodule type, nitrogen-fixing bacteroids and their free-living counterparts differ significantly. Mature (i.e., N_2-fixing) bacteroids are nondividing entities which metabolism is focused on securing the activity of

the nitrogenase enzyme and the transport of the resulting ammonium to the host (for reviews, see Prell et al. 2009 and White et al. 2007). In exchange for reduced forms of nitrogen, plants feed bacteroids with dicarboxylic acids as carbon source and, at least in a number of symbiotic systems, with branched-chain amino acids such as isoleucine, leucine, and valine for which bacteroids have become symbiotic auxotrophs (Prell et al., 2010). In several indeterminate nodule-forming legumes of the galegoid clade such as *Medicago* or *Vicia*, differentiation into N_2-fixing bacteroids has been shown to be irreversible and to result in the formation of enlarged endosymbiotic bacteria with endoreduplicated genomes (Mergaert et al., 2006). Terminal bacteroid differentiation is not necessarily a characteristic of indeterminate nodules however, as most bacteria isolated from nodules of *Mimosa pudica* have been shown to be capable of resuming free-living growth (Marchetti et al., 2011). In contrast to nondividing bacteroids, rhizobia that colonize the rhizoplane of host plants divide actively using as nutrients the abundant and chemically diverse root exudates. These free-living rhizobia are generally prototrophs for all amino acids, and their metabolism is not constrained to dicarboxylates as sole carbon source. In spite of these significant differences in lifestyles, the MALDI-TOF MS spectra of endosymbiotic and free-living forms of rhizobia were sufficiently similar to obtain an accurate identification (Ziegler et al., 2012).

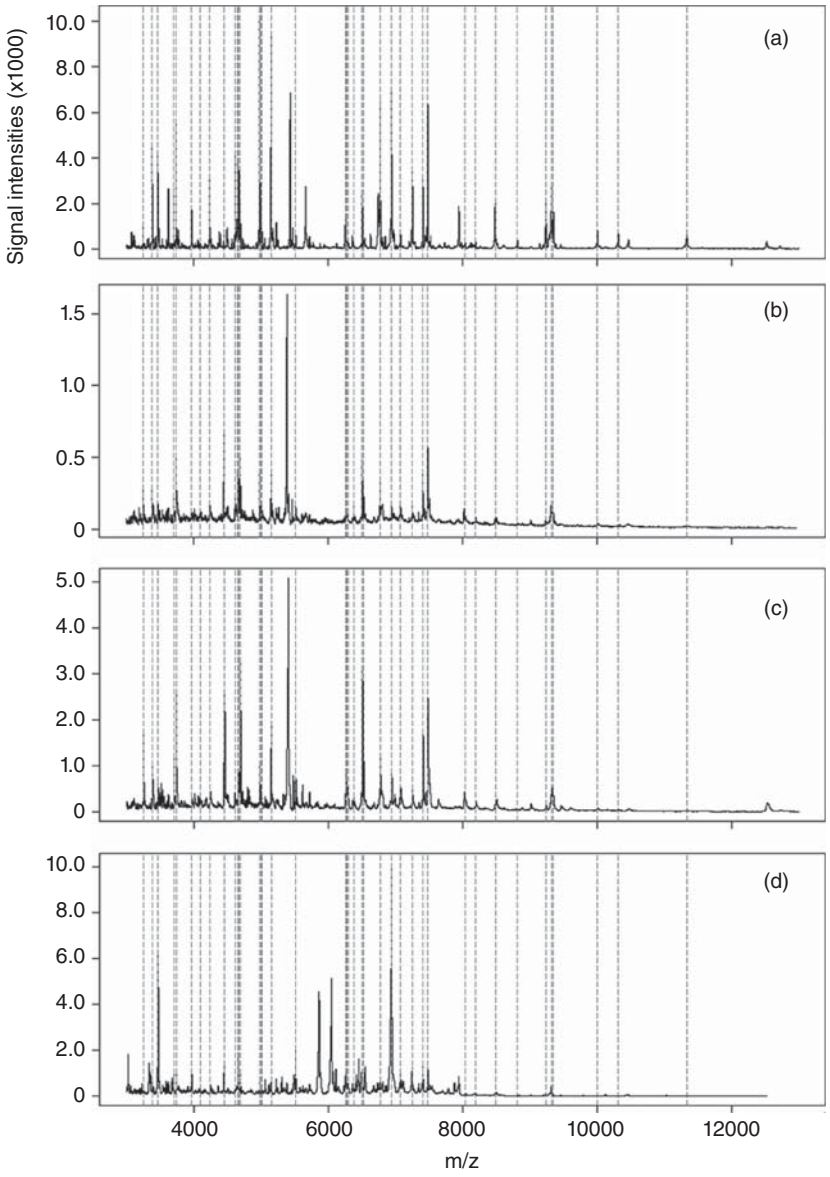

Figure 113.1 *Representative MALDI-TOF mass spectra of free-living or endosymbiotic cells* S. fredii *strain NGR234*. Recorded masses ranged from m/z 3000 to m/z 13,000, with the positions of biomarkers selected for computing the superspectrum of NGR234 shown as dashed lines. Cells of NGR234 were isolated from (a) a colony grown for 2 days on a TY plate at 28°C or nodules of (b) *V. unguiculata*, (c) *M. atropurpureum*, or *L. leucocephala* (d) collected, respectively, 42, 49, and 56 days postinoculation.

113.4.2 Identification of Bacteroids Isolated from Distinct Nodule Types

Initial results obtained with strains *S. fredii* NGR234, *S. fredii* USDA257, and *Bradyrhizobium japonicum* G49 indicated that MALDI-TOF MS-based identification of bacteroids was not compromised by the bacteroid-specific synthesis of the nitrogenase and accessory proteins that occurred inside *Vigna unguiculata*, *Macroptilium atropurpureum*, or *Glycine max* nodules of the determinate type (Ziegler et al., 2012). To test whether MALDI-TOF MS could reliably identify bacteroids found inside indeterminate nodules, plants of *Leucaena leucocephala* [Lam.] de Wit were inoculated with NGR234 and nodules collected 56 days postinoculation [dpi]. *L. leucocephala* is a small tropical tree native of Mexico that is cultivated for production of firewood and fibers as well as for its beneficial properties as green manure (Brewbaker, 1989). Analysis of the morphological and physiological characteristics of NGR234 cells isolated from nitrogen-fixing nodules of *L. leucocephala* using Percoll gradients showed that bacteroids [i] were of size and shape similar to free-living cells of NGR234, [ii] were actively respiring, [iii] had membranes that were permeable to propidium iodide [PI], and [iv] were in the vast majority [ca. 98%] incapable of resuming free-living growth (Bakkou, 2011). As shown in Figure 113.1, spectra generated from cells of NGR234 that were isolated from nodules of *V. unguiculata* (Fig. 113.1b), *M. atropurpureum* (Fig. 113.1c), or *L. leucocephala* (Fig.113.1d) shared a sufficient number of reference biomarkers (dotted lines in Fig. 113.1) for reaching a correct identification of bacteroids in the automatic mode. Thus, typing of NGR234 bacteroids was reliable independently of the host plant or nodule type. Unlike bacteroids of *Rhizobium leguminosarum* bv. *viciae* and *Sinorhizobium meliloti* which DNA contents

Table 113.1 The main characteristics of the rhizobial strains used in this study

Strain	Properties	Reference
1021	Str^R derivative of *Sinorhizobium meliloti* strain 2011	(Meade *et al.*, 1982)
3841	Str^R derivative of *Rhizobium leguminosarum* bv. *viciae* strain 300	(Johnston & Beringer, 1975)
AK83	*S. meliloti* strain isolated from *Medicago falcata* in Kazakhstan	(Giuntini *et al.*, 2005)
ANU265	Derivative strain of *Sinorhizobium fredii* NGR234 cured of pNGR234a	(Morrison et al., 1983)
BL225C	*S. meliloti* strain isolated from *Medicago sativa* in Italy	(Giuntini *et al.*, 2005)
BtAi1	Photosynthetic *Bradyrhizobium* strain isolated from a stem nodule of *Aeschynomene indica*	(Eaglesham & Szalay, 1983)
C58	*Agrobacterium tumefaciens* strain isolated in 1958 from a cherry tree tumor in New York State, USA	(Slater *et al.*, 2013)
CFN42	*Rhizobium etli* strain that forms N_2-fixing symbioses *Phaseolus vulgaris* isolated in Mexico	(Quinto *et al.*, 1982)
CIAT652	*R. etli* strain isolated in Costa Rica	(González *et al.*, 2010)
HH103	*S. fredii* strain that forms N_2-fixing symbioses with soybean and was isolated in China	(Dowdle & Bohlool, 1985)
LMG19424	*Cupriavidus taiwanensis* strain isolated from a nodule of *Mimosa pudica* in Taiwan	(Chen et al., 2001)
MAFF303099	*Mesorhizobium loti* strain isolated from *Lotus japonicus* in Japan	(Saeki & Kouchi, 2000)
ORS278	Strain of *Bradyrhizobium* sp. found in a stem nodule of *Aeschynomene sensitiva* in Senegal	(Lorquin *et al.*, 1997)
ORS571	*Azorhizobium caulinodans* strain from a stem nodule of *Sesbania rostrata* in West Africa	(Dreyfus & Dommergues, 1981)
ORS2060	*Methylobacterium nodulans* strain isolated from a nodule of *Crotalaria podocarpa* in Senegal	(Samba *et al.*, 1999)
NGR234	Rif^R derivative of the promiscuous *S. fredii* strain initially isolated from *Lablab* by M. J. Trinick	(Stanley *et al.*, 1988)
S4	*Agrobacterium vitis* strain isolated in 1981 by E. Szegedi from *Vitis vinifera* in Hungary	(Slater *et al.*, 2009)
USDA110	*Bradyrhizobium japonicum* strain isolated in 1959 from a nodule of soybean grown in Florida, USA	(Mathis *et al.*, 1997)
WSM419	Acid-tolerant strain of *S. meliloti*	(Howieson & Ewing, 1986)
WSM1325	*Rhizobium leguminosarum* bv. *trifolii* strain isolated from clover roots in Serifos, Greece	(Howieson *et al.*, 2005)
WSM2304	*R. leguminosarum* bv. *trifolii* strain from a nodule of *Trifolium polymorphum* in Uruguay	(Howieson *et al.*, 2005)

reached 18C and 24C, respectively (Mergaert et al., 2006), NGR234 bacteroids found inside *L. leucocephala* were 2C (Bakkou, 2011). This finding suggested that although a terminal differentiation process occurred inside nodules of *L. leucocephala*, it did not involve endoreduplication. Whether the endoreduplication process experienced by rhizobia inside *Medicago*, *Pisum*, or *Vicia* plants (Mergaert et al., 2006) affects the quality of MALDI-TOF MS spectra and the subsequent identification of bacteroids remains to be examined.

113.4.3 Toward a Rhizobia-Specific SARAMIS Database

The ability to correctly identify a bacterial strain via MALDI-TOF MS is influenced by a number of factors including the quality of the sample analyzed, the number of entries in the reference database, and the choice of biomarkers that together constitute the reference spectra (e.g., the so-called superspectra in the SARAMIS database) (Table 113.2). As discussed previously, current databases have been primarily designed and built for typing pathogenic bacteria (Martiny et al., 2012). With the aim to develop a database susceptible to facilitate the identification of rhizobia found inside plant nodules, we have begun to collect a number of well-characterized strains, selected to represent each of the genera of α- and β-proteobacteria that include symbiotic bacteria (Masson-Boivin et al., 2009). Figure 113.2 shows a cluster analysis of fingerprint spectra from 20 plant-interacting bacterial strains of the *Agrobacterium*, *Azorhizobium*, *Bradyrhizobium*, *Cupriavidus*, *Mesorhizobium*, *Methylobacterium*, *Rhizobium,* and *Sinorhizobium* genera. Although the structure of the resulting tree is not necessarily concordant with that of a phylogenetic tree based on 16S rDNA sequences, strains that belonged to the same species (e.g., S. *fredii* strains HH103 and NGR234 or the *S. meliloti* strains AK83, BL225C, and 1021) clustered together. This suggests that even though a fully exhaustive rhizobial database is unlikely to be assembled in the near future, unknown strains isolated from nodules are likely to be correctly typed provided spectra from a few members of the corresponding species are included in the reference MALDI-TOF MS database. In this respect, strains from the GEBA-RNB project are in the process of being integrated into the SARAMIS database. While the Genomic Encyclopedia of Bacteria and Archaea

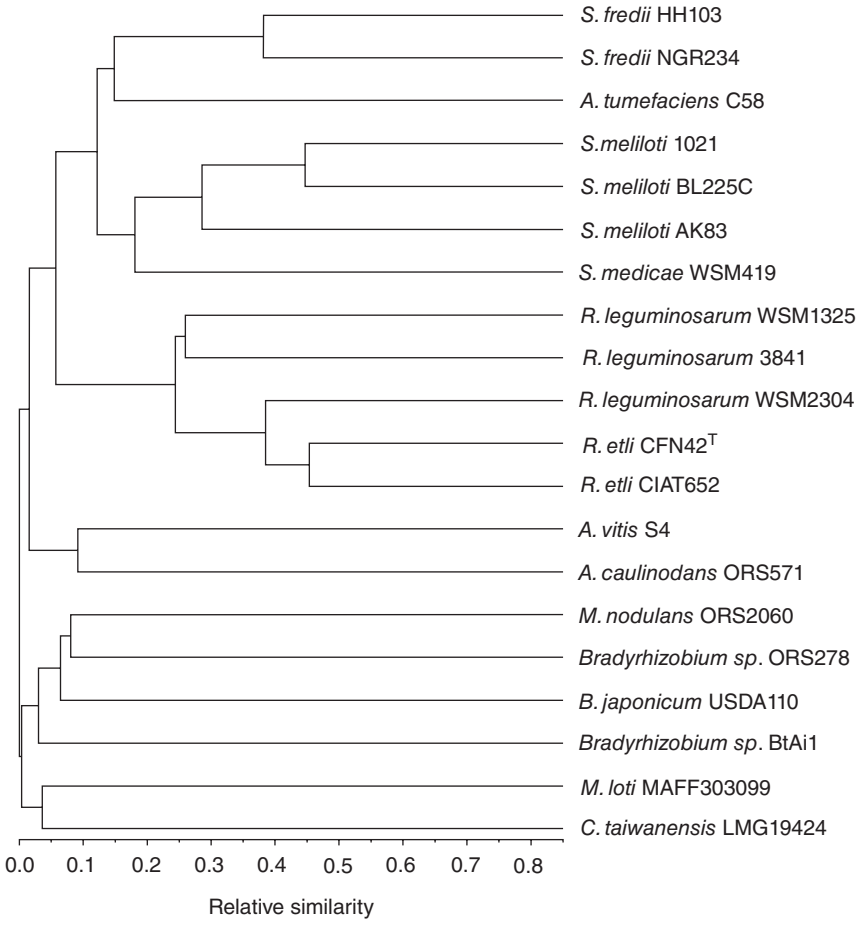

Figure 113.2 *Unsupervised hierarchical cluster analysis of spectra from 20 reference plant-interacting bacterial strains*. Spectra for each individual strain were obtained as described in the methods section, using free-living cells grown on TYA or YMA for one to five days at 28°C. Fingerprint spectra were subsequently assembled from this dataset and analyzed for similarity. Characteristics and origins of the listed strains are summarized in Table 113.1.

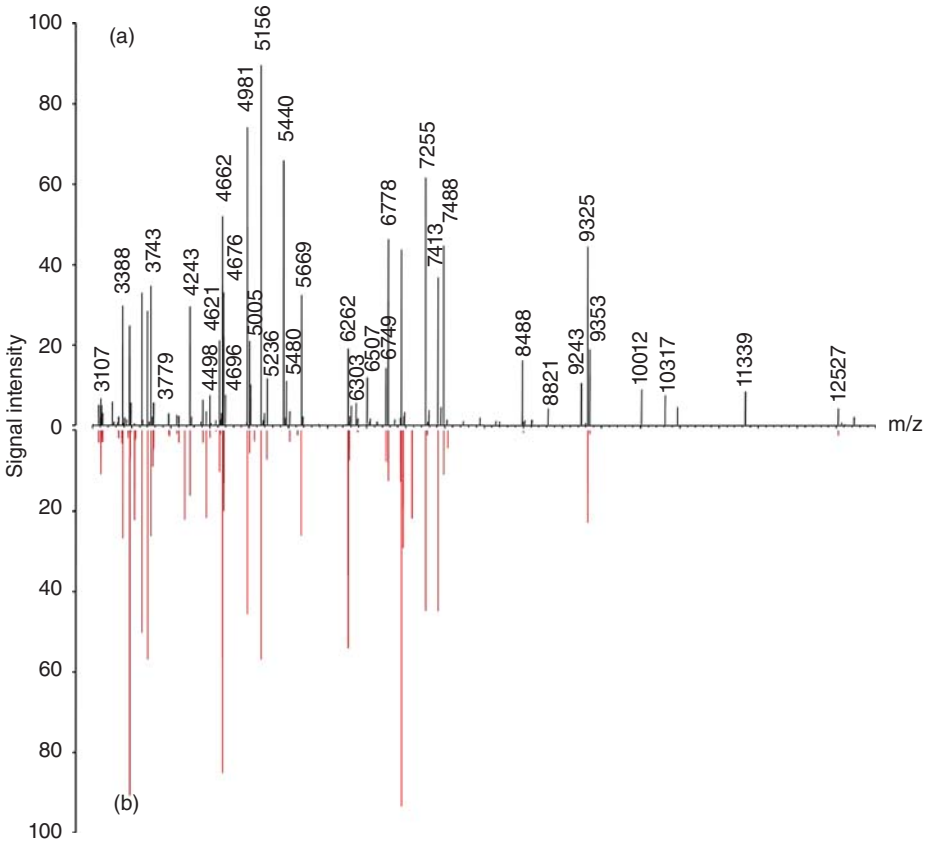

Figure 113.3 *Comparing the MALDI-TOF MS spectra of* S. fredii *strains NGR234 and ANU265*. Mirror display of representative spectra of two-day-old cells of NGR234 (a) or ANU265 (b) grown on TYA at 28°C. Recurrent masses in the range of 3000–13000 m/z that were selected as discriminative biomarkers for NGR234 were labeled according to their respective m/z. In the automatic mode, the matching spectra of ANU265 (one of which is shown in red) was identified as that of NGR234.

Table 113.2 Characteristics of a few leghemoglobins deposited in GenBank

Accession	Gene	Plant	Var./cv.	Size a.a.	Da	Tissue	Reference
Q43296	LbII	*Vigna unguiculata*	*California* n°5	145	15,350	Root nodule	(Arredondo-Peter *et al.*, 1997)
Q43236	LbI	*V. unguiculata*	*California* n°5	145	15,364	Root nodule	(Arredondo-Peter *et al.*, 1997)
P02234	LbA	*Phaseolus vulgaris*	*Kaiser Wilhelm*	146	15,618	Root nodule	(Lehtovaara & Ellfolk, 1975)
BAE46736	LjLb1	*Lotus japonicus*	*Miyakojima* MG20	146	15,346	Root nodule	(Uchiumi *et al.*, 2002)
BAE46737	LjLb2	*L. japonicus*	*Miyakojima* MG20	146	15,390	Root nodule	(Uchiumi *et al.*, 2002)
BAE46738	LjLb3	*L. japonicus*	*Miyakojima* MG20	147	15,754	Root nodule	(Uchiumi *et al.*, 2002)
BAE46739	LjHb1	*L. japonicus*	*Miyakojima* MG20	161	18,036	Whole plant	(Uchiumi *et al.*, 2002)
P27992	Lb1	*Medicago truncatula*	*Jemalong*	147	15,841	Root nodule	(Gallusci *et al.*, 1991)
P27993	Lb2	*M. truncatula*	*Jemalong*	146	15,752	Root nodule	(Gallusci *et al.*, 1991)

(GEBA) aims at sequencing many bacterial and archaeal genomes from diverse branches of the tree of life (see http://genome.jgi.doe.gov/programs/bacteria-archaea/GEBA.jsf), the root-nodulating bacteria (RNB) initiative that is coordinated by Wayne Reeve from Murdoch University has assembled more than 100 rhizobia from 15 different countries. That the genomes of these selected rhizobia will be completed soon is bound to facilitate the *in silico* analysis and validation of the MALDI-TOF MS reference spectra generated for each of these strains.

113.4.4 MALDI-TOF MS-Based Typing of Bacteroids: Strengths, Limitations, and Future Prospects

Together, these results confirm that identification of nodule bacteria via MALDI-TOF MS is rapid and accurate and successfully bypasses the time-consuming steps of isolating pure cultures and genomic DNA of rhizobia. In addition, resolution of MALDI-TOF MS is sufficient to reliably distinguish between the *S. fredii* strains NGR234, USDA257, and HH103, the genomes of which have been shown to be

remarkably similar (Schuldes et al., 2012, Weidner et al., 2012). Although a MALDI-TOF MS-based signature of a strain consists of a specific combination of more than 25 m/z biomarkers, it is unlikely to fully characterize the complexity of the 5,000 to 9,000 protein-coding genes of an average rhizobial genome. For example, we were not able to discriminate between *S. fredii* strains ANU265 and NGR234 using MALDI-TOF MS in the automatic mode (see Fig. 113.3). Derivative strain ANU265 was obtained by heat curing the 536 kb symbiotic plasmid pNGR234a (Morrison et al., 1983), thus deleting the NGR234 genome of ca. 8% of its coding capacity. ANU265 is incapable of nodulating host plants (Morrison et al., 1983) because it lacks the nodulation genes encoded by pNGR234a (Freiberg et al., 1997). RNA-Seq analyses confirmed that in cells of NGR234 grown in *Rhizobium* minimal medium supplemented with succinate (RMS), less than 10 of the >400 annotated genes of pNGR234a were transcribed (for more details, see Chapter 65 by A. Huyghe N. Bakkou and X. Perret). As none of these RMS-expressed genes of pNGR234a coded for proteins of 3 to 13 kDa, MALDI-TOF MS spectra of NGR234 did not include pNGR234a-specific biomarkers, and cells of ANU265 and NGR234 were not recognized as having distinct genomes. Most DNA-based genotyping protocols such as MLSA would not discriminate between NGR234 and ANU265 either, since the commonly used marker genes (e.g., *dnaK*, *glnA*, *gyrB*, *recA,* and/or *rpoB*) are chromosome born in both of the strains and of many other rhizobia.

Current protocols for preparing bacteroids for MALDI-TOF MS analysis include centrifugation and washing steps for removing leghemoglobins (Ziegler et al., 2012). Symbiotic leghemoglobins are small proteins of 12–16 kDa (see Table 113.2) that constitute up to 30% of the total content of soluble proteins in N_2-fixing nodules (Baulcombe and Verma, 1978). Together with a cortical oxygen diffusion barrier that surrounds the infected tissue of a functional nodule, leghemoglobins help regulate the oxygen tension within infected plant cells, and their synthesis is essential for an effective nitrogen fixation (Ott et al., 2009, 2005). Because of their abundance inside nodules and predominant protonation during sample ionization, symbiotic leghemoglobins mask other biomarkers in MALDI-TOF MS spectra unless removed from bacteroid preparations. Leghemoglobins should not be necessarily considered as undesirable contaminants, however. Instead, these abundant nodule proteins can be used as another set of biomarkers in MALDI-TOF MS analyses of nodules in order to confirm the identity of the legume host. In field studies, the roots of selected host plants generally need to be carefully exposed and cleared of soil before nodules that are still attached to these roots can be collected. This practice that reduces the chances of collecting nodules from a neighboring host makes current field studies slow, labor-intensive, and often difficult to conduct on a large scale. By combining the successive MALDI-TOF MS signatures of leghemoglobins and bacteroids, a binary nodule-typing method would make large-scale field studies of detached nodules both faster and easier to conduct. Together with an improved and robust rhizobial-specific SARAMIS^{TM} database, such a protocol of identification for both plant and microbial symbionts should improve our understanding of rhizobia–legume interactions and evolutions in ecological niches as diverse as inoculated soybean fields, natural prairies, or rain forests.

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Chapter 114

The Microbe-Free Plant: Fact or Artifact

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114.1 INTRODUCTION

114.1.1 Endophytes: A Hidden Diversity

Two of the most well-known and most intensively studied plant–microbe interactions are the mutualistic associations that are formed by plant roots with mycorrhizal fungi and rhizobia. Besides these nutritional mutualisms, however, most plant–microbe interactions are considered detrimental for the host. Roots, shoots, leaves, flowers, and fruits of all plants are regularly infected by bacteria, fungi, and viruses, which act as pathogens and cause disease when not successfully controlled by the host resistance system. Plant pathogens can considerably decrease the fitness of their host or, when it comes to crops, yield. Although pathogens can increase plant diversity in natural ecosystems due to their "Janzen–Connell" effects (Janzen, 1970; Connell, 1971; Garcia Guzmán and Heil, 2014), we perceive them usually like an agronomist or medical doctor: microbial infection in a plant is considered as undesirable.

However, like we just begin to realize the ubiquity and beneficial role of the human microbiome (e.g., intestinal bacteria), research over the last decade has discovered that plants are full of fungi, bacteria, and viruses even when they look phenotypically healthy, that is, express no symptoms of disease (Wilson, 1995; Arnold et al., 2000; Yuan et al., 2010; Partida-Martinez and Heil, 2011; Porras-Alfaro and Bayman, 2011). The so-called endophytic fungi have been isolated from all plant organs and are usually found in 80–100% of the investigated samples. Multiple taxa can commonly be isolated from a single host species or even a single leaf. For example, fungal species representing 18 taxonomic orders were reported from symptomless and surface-sterilized switchgrass (*Panicum virgatum* L.) (Ghimire et al., 2011), 100 morphologically distinct groups in 33 taxa of endophytic fungi were discovered in leaves of European aspen (*Populus tremula*) (Albrectsen et al., 2010), 60 taxa were reported from leaves and twigs of *Quercus ilex* (Fisher et al., 1994), 58 operational taxonomic units (OTUs) were isolated from leaves of wild rubber trees (*Hevea brasiliensis*) in Peru (Gazis and Chaverri, 2010), more than 100 morphospecies of endophytes were found to be associated with tropical palms (Frohlich et al., 2000), and 418 fungal morphospecies could be isolated from 83 leaves from different tropical tree species (Arnold et al., 2000). Whereas most studies on fungal endophytes focused on broad-leaved plants from tropical and temperate systems, many other types and growth forms and habitats remain to be screened. Recent studies reported, for example, 64 species of fungal endophytes from marine algae (Flewelling et al., 2013) and 59 taxa from the cactus *Cereus jamacaru* (Bezerra et al., 2013).

Besides fungi, bacteria represent the second largest group of plant endophytes. Bacterial endophytes seemingly are at least as diverse and widespread as fungal endophytes. Traditionally, symbiotic bacteria are mainly investigated in the context of the rhizosphere, and thus, most reports focus on soil-derived bacteria (Pieterse et al., 2001; Pineda et al., 2010; 2013; de Bruijn, 2013). Many of these bacteria colonize the root tissue and then live as endophytes. For example, 256 strains of root-colonizing bacteria were found

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in surface-disinfected root tissues of six plant species in Korea (Bibi et al., 2012). However, endophytic bacteria are not restricted to the root but rather infect all parts of a plant and can be found in all types of plants. For example, 283 endophytic bacterial isolates were obtained by culture-based methods from *Sphagnum fallax* (Shcherbakov et al., 2013), most of which were identified as *Burkholderia*, *Pseudomonas*, *Flavobacterium*, *Serratia*, and *Collimonas* (Shcherbakov *et al.*, 2013). A nonculture-based approach (pyrosequencing of 16S rRNA gene amplicons) detected >850 bacterial OTUs in leaves of spinach (*Spinacia oleracea*) (Lopez-Velasco et al., 2013). Because the seeds in this study contained "only" *ca.* 250 different OTUs from the Proteobacteria, Firmicutes, and Actinobacteria, the authors conclude that horizontal acquisition plays an important part in the assembly of this rich bacterial community. Like endophytic fungi, endophytic bacteria seemingly have been detected in all plants that have been screened for them, including desert plants such as *Plectranthus tenuiflorus* [28 bacterial endophytic isolates from different organs comprised *Bacillus*, *Micrococcus*, *Paenibacillus*, *Pseudomonas*, and *Acinetobacter*; see El-Deeb et al., 2013], the arctic moss (*Sanionia uncinata*) (Park et al., 2013), and the arctic higher plants *Oxyria digyna*, *Diapensia lapponica*, and *Juncus trifidus* (Nissinen et al., 2012). Finally, bacteria can also inhabit the hyphae of fungal endophytes and then live as "endo-endophytes" (Hoffman and Arnold, 2010).

Species saturation curves demonstrate that all these studies were unlikely to discover all endophytes present in the investigated host plants (Arnold et al., 2000; Frohlich et al., 2000; Higgins et al., 2011). Even new investigations of standard model plants such as *Arabidopsis* and maize reveal an as-yet-unknown diversity of endophytes (Liu et al., 2012; Bodenhausen et al., 2013). Thus, we must assume that we significantly underestimate the diversity of endophytes and, hence, their potential importance in natural ecosystems. Many more datasets will be required to obtain insights into the general association patterns among endophytes and their host plants and to formulate testable hypotheses concerning the underlying mechanisms of colonization.

114.1.2 Defining "Endophytes"

Endophytes have been defined by Wilson (1995) as "fungi that are present in host plant tissues, during at least part of their life cycle, without causing visible symptoms." Unfortunately, recent evidence has revealed some major drawbacks of this definition. First, microorganisms that develop within living plant tissue without causing visible symptoms are not restricted to fungi but also comprise bacteria and viruses. Second, the net effects of infection on the host are highly conditional and can shift from mutualism to antagonism for virtually any type of plant–microbe interaction (see discussion and references in Partida-Martinez and Heil, 2011). Multiple strains of classical endophytes have been reported to shift at an ecological or evolutionary timescale from endophytic to pathogenic behavior and then usually act as necrotrophs (Delaye et al., 2013). Thus, the term "endophyte" does not necessarily describe a fixed life history trait but refers to a specific microorganism in a specific host under a specific set of environmental conditions.

In this chapter, I use the term **symptomless endophyte** to denominate all microorganisms that develop within living plant tissue and cause no disease. This definition is highly conditional. Furthermore, **symptoms** in this context refer explicitly to **symptoms of disease**. Although symptomless endophytes cause no disease symptoms, they influence the phenotype and epigenome of their host plant (Gilbert et al., 2010). In fact, significant parts of the plant phenotype can represent the **extended phenotype** sensu Dawkins (1999) of one or several microorganisms (Porras-Alfaro and Bayman, 2011). For example, even the production of essential oils in medicinal plants can depend on the colonization by specific endophytes (Ren and Dai, 2012), and Taxol, a **classical** defensive metabolite of Pacific yew (*Taxus brevifolia*), is synthesized by an endophytic fungus rather than by the host plant itself (Stierle et al., 1993). An endophyte-free plant looks and functions differently from an endophyte-infected one under most natural growing conditions (Figure 114.1). It is their omnipresence rather than the lack of visible effects which led earlier researchers to the conclusion that

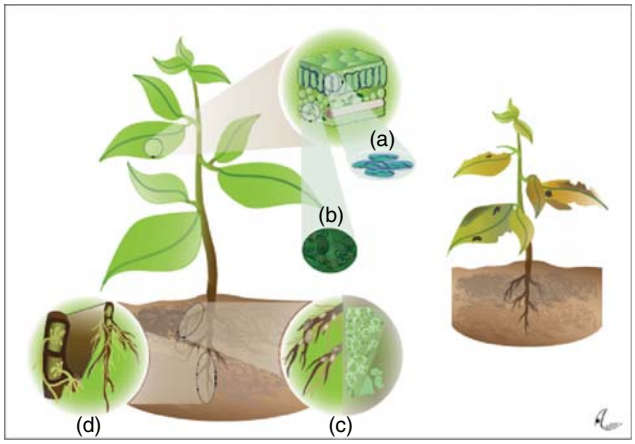

Figure 114.1 *Phenotypic effects of "symptomless" endophytes.* Endophytes such as fungal leaf endophytes (a), bacterial leaf endophytes (b), rhizobia (c), and arbuscular mycorrhizal fungi (d) can colonize all organs of a plant. Due to the ubiquity of these endophytes, the phenotype that we usually know as the normal phenotype of the plant (left panel) represents in fact the phenotype of the halobiont. Endophytes affect so many vitally important traits of their host that a completely endophyte-free plant would suffer from chlorosis and lowered photosynthetic rates due to nutritional shortcomings, lowered resistance to abiotic stress and lowered resistance to both pathogens and herbivores, and, in consequence, lowered competitive abilities and lower survival rates in nature (right panel). Figure from Partida-Martinez and Heil (2011).

endophytes do not cause visible symptoms (Wilson, 1995). The extended phenotype of the endophytes is easily overseen because it represents an integrative, ubiquitous part of the plant phenotype that we usually see (Partida-Martinez and Heil, 2011). Here, I summarize the most commonly reported effects that endophytes have on the resistance in their host plants to biotic stress. Considering their frequently positive effects on the resistance level of their host, I then will discuss the potential of phenotypes in agriculture.

114.1.3 Classes of Endophytes

The first fungal endophytes were discovered in certain cultivars of tall fescue (*Lolium arundinaceum*) and of related grasses (mainly of the genera *Festuca* and *Lolium*) that cause livestock disorders. In these grasses, asexual, alkaloid-producing fungi of the genus *Neotyphodium* could be identified as the causal agent of their negative effects on mammal and insect herbivores (see Clay, 1990, and references cited therein). These "type I fungal endophytes" sensu Yuan et al. (2010) are a taxonomically well-defined group in the family Clavicipitaceae that are vertically transmitted via the seeds of their hosts and therefore cause systemic infection of certain grasses. They directly contribute to the resistance of their host plants to herbivores, but numerous studies have also reported effects on drought and flooding tolerance, biomass production, and – in consequence – the competitive ability of the host grass (see Lehtonen et al., 2005; Schulz and Boyle, 2005, for references).

Less studied but perhaps much more diverse and ubiquitous are the so-called type II fungal endophytes (Yuan et al., 2010). These fungi normally are nonsystemic, horizontally transmitted species that colonize de novo every single leaf. Numerous studies reported surprisingly high numbers of taxa and infection rates by these **nonclavicipitaceous** fungal endophytes. Type II fungal endophytes comprise species from both Ascomycota and Basidiomycota, although Ascomycota are usually dominating (Fisher, 1992; Frohlich et al., 2000; Arnold and Herre, 2003; Albrectsen et al., 2010).

Bacterial endophytes represent a taxonomically and functionally highly diverse group that infects roots and shoots (Weyens et al., 2009). Together with rhizosphere bacteria, these bacteria form the large functional group of "plant growth-promoting bacteria (PGPB)" (see de Bruijn, 2013), although evidence is accumulating that they might contribute to the resistance of their host as commonly as do fungal endophytes.

114.2 PHENOTYPIC EFFECTS OF ENDOPHYTES

114.2.1 Growth Promotion

A very rich literature has described the positive effects on plant growth of PGPB (Pieterse et al., 2001; Zehnder et al., 2001a, 2001b; Brock et al., 2013; de Bruijn, 2013). Having been isolated originally from the rhizosphere, many of these bacteria also colonize the root tissue and then must be considered as endophytes, just as it is true for rhizobia and most mycorrhizal fungi (arbuscular mycorrhiza and endomycorrhiza). Whereas plant–microbe interactions are, thus, most commonly studied belowground, many diazotrophic endophytic bacteria such as *Acetobacter*, *Azospirillum*, *Azoarcus*, *Herbaspirillum*, *Klebsiella*, *Nostoc*, and *Pantoea* can colonize the aerial parts of a plant (leaves and shoots) and are commonly reported to fix nitrogen (Dobereiner, 1992; James and Olivares, 1998; Remus et al., 2000; Krause et al., 2006; Schreiner et al., 2009; Yasuda et al., 2009; Brock et al., 2013; Santi et al., 2013). In fact, N-fixation by endophytic plants "outside the legumes" appears to be a severely underestimated process that readily contributes to the N-supply of natural and agronomic ecosystems (Santi et al., 2013). Endophytic bacteria that colonize root tissues secrete compounds that enhance nutrient uptake by phosphate solubilization or act as siderophores and thus enhance iron chelation. In fact, a single endophyte strain can exert multiple functions. For example, the PGPB *Pseudomonas stutzeri* RP1, which colonized roots of sunflower (*Helianthus annuus*), showed antibacterial activity against *E. coli*, *Xanthomonas* sp., *Serratia* sp., *Bacillus* sp., and *Pseudomonas* sp. and also had growth-promoting attributes such as phosphate solubilization and the production of indole acetic acid and NH_3 and antiherbivore defensive potential via the production of HCN (Pandey et al., 2013). The growth-promoting effects of endophytes can also reduce any negative effects on fitness that might result from damage caused by pathogens or herbivores. In this case, endophytes cause **tolerance** to biotic stressors. For example, clavicipitaceous endophytes might reduce the negative effects of viral infection via changing biomass allocation to roots (Rúa et al., 2013).

114.2.2 Resistance to Infection

An increased resistance to pathogens appears to be a common consequence of plant infection or association with PGPB (Pieterse et al., 2001; Pieterse and Dicke, 2007) but is also reported for plants that are infected with rhizobia or mycorrhiza (Borowicz, 2001; Pozo and Azcón-Aguilar, 2007; Hartley and Gange, 2009; Pineda et al., 2010). Root-colonizing PGPB commonly mediates the plant's own resistance traits (i.e., systemic signaling; see Pieterse et al., 2001), but also the colonization of the aerial parts of a plant by endophytes can enhance the resistance in leaves to pathogens. The first report on pathogen resistance mediated by type II endophytes appears to be the observation that inoculation of leaves of cacao (*Theobroma cacao*) seedlings with fungal endophytes, which had been isolated from naturally colonized asymptomatic hosts, significantly decreased

Table 114.1 Effects of type II endophytes on the resistance in their host plant to biotic stress*

Endophyte Group	Stress Type	Endophyte	Host	Effect	Type of Pathogen	Reference
Fungi	*Pathogens*	*Stachybotrys* sp., *Trichoderma atroviride*, *Ulocladium atrum,* or *Truncatella angustata*	*Populus* sp.	Local resistance to rust caused by *Melampsora x columbiana*	Basidiomycota [biotroph]	(Raghavendra and Newcombe, 2013)
		Fusarium verticillioides	*Zea mays*	Resistance to corn smut disease when endophyte is coinoculated with *Ustilago maydis*	Basidiomycota [biotroph]	(Lee et al., 2009)
		Trichoderma stromaticum	*Theobroma cacao*	Resistance in seedlings to the witches' broom pathogen [*Moniliophthora perniciosa*]	Basidiomycota [hemibiotroph]	(De Souza et al., 2008)
		Mixture of seven endophyte strains	*T. cacao*	Reduced symptoms caused by *Phytophthora* sp.	Oomycota [necrotroph]	(Arnold et al., 2003)
	Herbivores	*Glomerella cingulata*	*Merremia umbellata*	Reduced reproduction by adult *Chelymorpha alternans* beetles, which as larvae had fed on leaves with high endophyte load	Coleoptera	(Van Bael et al., 2009)
		Acremonium strictum	*Vicia faba*	Impaired fitness of *Helicoverpa armigera*	Lepidoptera	(Jaber and Vidal, 2010)
		Chaetomium cochliodes	*Cirsium arvense*	Reduced feeding by the generalist herbivore *Mamestra brassicae*	Lepidoptera	(Gange et al., 2012).
		Nigrospora sp. and *Cladosporium* sp.	*Brassica oleracea*	Enhanced mortality of the generalist *Spodoptera litura*	Lepidoptera	(Thakur et al., 2013)
		Fusarium oxysporum Fo162	*Cucurbita pepo*	Enhanced resistance to *Aphis gossypii*	Hemiptera	(Martinuz et al., 2012)
Bacteria	*Pathogens*	*Bacillus megaterium*, *Salmonella enterica*, *Brevibacillus choshinensis*, *Cedecea davisae*, *Microbacterium testaceum*, *Pectobacterium carotovorum*	*Coffea arabica*	Resistance in leaf disks to leaf rust caused by *Hemileia vastatrix*	Basidiomycota [biotroph]	(Silva et al., 2012)
		Bacillus sp.	*T. cacao*	Black pod rot [*Phytophthora capsici*], oomycete	Oomycota [hemibiotroph]	(Melnick et al., 2008)
		Azospirillum sp. B510	*Oryza sativa*	*Magnaporthe oryzae* [fungus]	Ascomycota [hemibiotroph]	(Yasuda et al., 2009)
		Azospirillum sp. B510	*O. sativa*	*Xanthomonas oryzae* [bacterium]	Proteobacteria [biotroph]	(Yasuda et al., 2009)
	Herbivores					
		Rhizobium etli G12	*C. pepo*	Enhanced resistance to *A. gossypii*	Hemiptera	(Martinuz et al., 2012)

*I present a noncomprehensive list of recent examples of how colonization by nonclavicipitaceous (type II) fungal endophytes or by bacterial leaf endophytes can affect the resistance of their host plants at the phenotypic level. Please see Saikkonen et al. (1998, 2010) for reviews on effects on clavicipitaceous endophytes; Gao et al. (2010), Ownley et al. (2010), and Yuan et al. (2010) for reviews on effects of nonclavicipitaceous fungal endophytes; and Sturz et al. (2000) and Pineda et al. (2010) for reviews on resistance-enhancing effects of soilborne endophytic bacteria.

both necrosis and mortality of the leaf when the seedlings were challenged with a pathogenic *Phytophthora* sp. (Arnold et al., 2003). Reports published over the following years indicate that an enhanced resistance to pathogens might be a common outcome of the colonization of plants by fungal or bacterial symptomless endophytes (van der Lelie et al., 2009; Weyens et al., 2009; Gao et al., 2010; Ownley et al., 2010) (Table 114.1).

114.2.3 Resistance to Herbivores

The ecological role of the clavicipitaceous endophytes has been discovered via their lethal effects on herbivorous mammals (Bacon et al., 1977; Saikkonen et al., 1998; Faeth, 2002). Subsequent studies demonstrated that the colonization of grasses by these fungi also enhances their resistance to multiple chewing and sucking insects (Kuldau and Bacon, 2008), and a recent meta-analysis revealed that colonization of host grasses by these fungi usually enhances their resistance to herbivores (Saikkonen et al., 2010). However, the species and the feeding mode of the herbivore clearly matter in this context. For example, experiments using the grass *Festuca subverticillata* and the type I endophyte *Neotyphodium* sp. revealed that the long-horned grasshopper *Pterophylla camellifolia*, the caterpillar *Spodoptera frugiperda*, and the aphid *Rhopalosiphum padi* preferred endophyte-free plants, whereas the short-horned grasshoppers *Encoptolophus costalis* and *Romalea guttata* preferred plants with the endophyte (Afkhami and Rudgers, 2009).

Whereas there exist numerous reports on antiherbivore effects of these type I endophytes (for reviews, see Saikkonen et al., 1998, 2006; Faeth, 2002; Rudgers et al., 2009; Porras-Alfaro and Bayman, 2011), much less is known on type II endophytes. Females of tropical beetles exhibited impaired reproduction when they had to feed on endophyte-colonized leaves as larvae (Van Bael et al., 2009). Similarly, broad bean *Vicia faba* plants that were colonized with the root endophytic fungus *Acremonium strictum* exhibited enhanced resistance to the generalist leaf herbivore *Helicoverpa armigera*. The endophyte could not be reisolated from the leaves, which makes a systemic effect likely (Jaber and Vidal, 2010). Colonization of cauliflower (*Brassica oleracea*) with the fungal endophytes *Nigrospora* sp. and *Cladosporium* sp. caused higher mortality of the generalist caterpillar *Spodoptera litura*, although these endophytes had been isolated from a different plant species (Thakur et al., 2013). Finally, even seemingly well-defended plants like thistle (*Cirsium arvense*) might benefit from reduced feeding by a generalist insect when their leaves are colonized by specific endophytic fungi (Gange et al., 2012).

By contrast, observations of mountain birches (*Betula pubescens* ssp. *czerepanovii*) over a 3-year period revealed little support for a direct relation between the frequencies of endophytic fungi and the performance of invertebrate herbivores (Ahlholm et al., 2002). In fact, evidence for antiherbivore effects in a meta-analysis was much less convincing for type II endophytes than for type I endophytes (Saikkonen et al., 2010). This observation can be interpreted in different ways. (i) The most conservative explanation would simply be that horizontally transmitted type II endophytes less frequently enhance host resistance to herbivores than type I endophytes. Because most of these endophytes are closely related to necrotrophs or even can shift to necrotrophic behavior (Delaye et al., 2013), the shared JA-dependent resistance pathways that are used by plants to fend off necrotrophic pathogens and chewing herbivores might be a causal explanation for this observation. (ii) Second, type II endophytes usually form diverse communities and no strain achieves domination (Arnold and Herre, 2003; Porras-Alfaro and Bayman, 2011). Therefore, each single fungal train might not reach sufficient density to exert any detectable effect on insects. (iii) Third, effects of high endophyte loads might differently affect the different developmental stages of insects, and of course, they vary among strains of endophytes and species of insects. For example, larvae of the beetle *Chelymorpha alternans* showed no preference for host leaves with low endophyte loads and did not suffer from any detectable effects in development or survival. However, female beetles that as larvae fed on host leaves with high endophyte loads produced 80% less offspring (Van Bael et al., 2009). The negative effect of endophytes on the fitness of *H. armigera* was even carried to a second generation that was maintained completely on an endophyte-free artificial diet (Jaber and Vidal, 2010). However, caterpillars in the first generation had to feed on intact plants, because no negative effects of endophyte colonization on the fitness of the insect were observed when the experiments were performed with leaf disks (Jaber and Vidal, 2010). In the study by Gange and colleagues, colonization by two out of the four endophyte strains tested increased rather than decreased feeding by a specialist insect herbivore (*Cassida rubiginosa*), and only one out of four endophytes tested (*Chaetomium cochliodes*) significantly reduced feeding by the generalist herbivore *Mamestra brassicae* (Gange et al., 2012). Effects of these types are likely to remain undiscovered in most assays, in which we usually focus on choice behavior or on immediate effects on consumption rates or larval growth rate and commonly use leaf disks rather than entire plants.

114.3 MECHANISMS: WHAT DO WE KNOW?

114.3.1 Growth Promotion

The growth-promoting effects of bacteria that colonize plant root or shoots as epiphytes or endophytes result from

multiple mechanisms such as (i) the direct acquisition and provision of nutrients (via N-fixation, the solubilization of inorganic phosphates, the production of siderophores that facilitate iron uptake, etc.), (ii) the production of plant hormones, (iii) the induction of resistance-related plant genes, and (iv) direct antibiosis against pathogens (Weyens et al., 2009; Dudeja et al., 2012; de Bruijn, 2013). Taxa that are frequently reported in this context are the genera *Pseudomonas* (in particular multiple strains of *Pseudomonas fluorescens*), *Bradyrhizobium*, *Azorhizobium*, *Azospirillum*, and *Bacillus* (commonly *Bacillus thuringiensis*). Different mechanisms commonly are exerted by the same microbial strain. For example, the growth-promoting endophytic bacterium *Enterobacter radicincitans* DSM 16656 fixes atmospheric nitrogen, solubilizes phosphate, produces phytohormones, and induces several resistance-related plant genes (Brock et al., 2013).

114.3.2 Resistance to Infection

Although different endophytes often cause phenotypically similar resistance to pathogen infection, this effect can be achieved by numerous direct and indirect mechanisms. Direct effects are particularly likely to exist when the resistance effect is locally restricted to the infected tissue. For example, *T. cacao* leaves that were infected with type II fungal endophytes showed a locally enhanced resistance to pathogens only in the infected areas, and direct *in vitro* confrontation assays demonstrated strong antagonisms between certain strains of endophytes and the pathogen (Arnold et al., 2003). Direct effects of endophytes on the phenotypic resistance of their host can result from the production of antibiotics; of extracellular lytic enzymes such as chitinases, cellulases, proteases, and ß-1,3-glucanases; and of secondary compounds, when endophytes act as mycoparasites and directly parasitize plant pathogenic fungi or when nonsymptomatic endophytes outcompete the pathogens (Weyens et al., 2009; Gao et al., 2010; Ownley et al., 2010; de Bruijn, 2013).

Belowground microorganisms are also very commonly reported to locally enhance the resistance in the roots (Borowicz, 2001; Pozo and Azcón-Aguilar, 2007; Elsen et al., 2008; Pineda et al., 2010), which makes it likely that similar mechanisms are involved. Root-colonizing bacteria or fungi might prevent pathogen invasion directly, for example, by outcompeting pathogens on the root surface or within the tissue or by the production of antifungal or antibacterial compounds (Haggag, 2010; de Bruijn, 2013). For example, 9% of the 256 strains of root-colonizing endophytic bacteria that were isolated from six plant species in tidal flats in Korea showed antagonistic behavior against plant pathogenic oomycetes *in vitro*, and many of these bacteria were found to produce ß-1,3-gucanases, chitinases, cellulases, and proteases (Bibi et al., 2012). Most of these traits have evolved in the context of antagonistic interactions among microorganisms and thus are unlikely to represent a specific adaptation of endophytes to their potential role as mutualists of their host. However, they invariably can benefit any host plant that is colonized by a competitively dominant microorganism with low virulence.

As a second mechanism, the infection of plants with endophytes that enhance the nutritional status of their host (mycorrhiza, rhizobia, and other PGPB) can decrease the plant internal competition among different traits for limited nutrients (Herms and Mattson, 1992). This effect alone might allow for systemically enhanced resistance levels (Van Dam and Heil, 2011). As a consequence, mycorrhiza can improve resistance to leaf pathogens (Fritz et al., 2006; Pozo and Azcón-Aguilar, 2007; Alejo-Iturvide et al., 2008), and also, plants whose roots are infected with rhizobia commonly show enhanced resistance levels in their aboveground parts. For a short overview on ecologically relevant resistance-enhancing effects of mycorrhiza and nodulation on aboveground communities, see Van Dam and Heil (2011).

Furthermore, indirect – and commonly systemic – effects result when endophytes trigger unspecific responses of the plant's own immune system. For example, the colonization by symptomless fungal endophytes induces the expression of resistance-related genes in their host (Mithöfer, 2002; Pozo and Azcón-Aguilar, 2007; Ownley et al., 2010). Similarly, the colonization of roots by mycorrhizal fungi commonly enhances resistance to necrotrophic leaf pathogens, whereas the resistance to biotrophic pathogens is often impaired. The most likely reason for this mycorrhiza-induced systemic resistance is a mycorrhiza-induced shift between the different signaling pathways that control plant resistance to necrotrophic versus biotrophic pathogens: these functional groups of pathogens are controlled by different signaling pathways that are subject to a negative trade-off. The mycorrhizal fungus, which itself lives as a biotroph, must suppress the resistance to biotrophs in order to colonize the plant and thereby enhance resistance to necrotrophs (Pozo and Azcón-Aguilar, 2007).

Also, PGPB that infect the aerial parts of plants can induce or prime resistance-related genes (Pieterse et al., 2001; Pineda et al., 2010). The endophytic bacterium *Pseudomonas putida* MGY2 enhanced gene expression (and also activity at the enzymatic level) of phenylalanine ammonia lyase, catalase, and peroxidase in the tissue of the host (Shi et al., 2011), another *Pseudomonas* strain induced both basal resistance and induced resistance dependent on SA and JA/ethylene signaling in potato (Pavlo et al., 2011), and the N-fixing and growth-promoting bacterium *E. radicincitans* induced various SA- and JA-dependent resistance genes in *Arabidopsis*, including PR1, PR2, PR5, and PDF1.2 (Brock et al., 2013). Thus, the induction of JA- or SA-dependent resistance genes appears a common effect of PGPB that colonize plant tissues. By contrast, colonization of rice (*Oryza*

sativa) with the N-fixing endophytic bacterium, *Azospirillum* sp. B510, enhanced resistance to rice blast disease caused by the fungus *Magnaporthe oryzae* without measurably affecting endogenous SA accumulation or PR gene expression and thus might function via an SA-independent pathway (Yasuda et al., 2009).

Due to the systemic nature of most resistance responses, it might not matter too much whether the resistance-inducing agent is colonizing belowground or aboveground tissues. Most importantly, all these phenomena are mediated by effects of the endophytes on host gene expression patterns and therefore represent the "extended phenotype" of the microorganisms, although they are likely to depend more strongly on host genotype than the direct effects by, for example, toxic fungal metabolites or fungal mycoparasites.

114.3.3 Resistance to Herbivores

From the day of their discovery as the causal reason of livestock disease, fungal endophytes were related to the antiherbivore defense of their host plant (see in the preceding text). The toxic effect on livestock of fungal alkaloids represents a direct antiherbivore defensive mechanism that the plants gain when being colonized by a type I endophyte. However, as described previously for pathogens, the enhanced resistance of endophyte-colonized host plants to herbivores can result from multiple direct and indirect mechanisms.

Although one might expect a positive effect due to the enhanced nutrient contents of mycorrhized plants, the performance of herbivorous insects on AMF-inoculated plants usually is often lower than on unmycorrhized plants (Hartley and Gange, 2009; Kempel et al., 2010; Pineda et al., 2010). Similarly, the colonization of roots by rhizobia can increase resistance in the aboveground parts of soybean to aphids (Dean et al., 2009). As explained previously, the suppression of the host's SA-dependent resistance to biotrophs and the resulting enhanced expression of JA-dependent resistance to herbivores and necrotrophs are likely to contribute to this effect.

Fungal symptomless leaf endophytes are likely to enhance the resistance to herbivores of their host plant via four distinct mechanisms: (i) via the production of alkaloids and other toxic compounds, (ii) due to their function as entomopathogens, (iii) via the induction or priming of resistance-related genes, and (iv) by enhancing the genetic and biochemical diversity of their host. As described previously for bacteria, these modes of action are not necessarily exclusive. For example, entomopathogenic fungal endophytes can also associate with the rhizosphere, act as antagonists of plant pathogens, and might possibly even function as plant growth-promoting agents (Vega et al., 2009).

A recent meta-analysis revealed that alkaloid-producing type I endophytes in general slightly enhance host resistance to insects, although this result might be biased due to the concentration of the experimental effort on few agronomically important model systems (Saikkonen et al., 2010). For type II endophytes, by contrast, the situation appears to be less clear. Beyond doubt, *in vitro* cultures of many type II endophytic fungi produce compounds with antibacterial, fungicidal, and herbicidal effects (for references, see Schulz and Boyle, 2005; Gao et al., 2010; Ownley et al., 2010), and these compounds could exert a direct resistance effect. Type II endophytes, however, in general reach much lower densities in their host than do type I endophytes. In European aspen (*P. tremula*), the phenotypic resistance to herbivores of different clones was positively correlated to their load of endophytic fungi (Albrectsen et al., 2010), which makes a direct effect likely that is mediated by some fungal metabolites. A very specific case of antiherbivore effect exerted by type II endophytes is represented by the discrimination exhibited by leaf-cutter ants (*Atta colombica*) against leaves with higher endophyte colonization rates. In this case, the most likely explanation is a negative effect of the endophytes on the growth of the fungus in the ants' gardens (Van Bael et al., 2012).

Second, several plant endophytic fungi also live as entomopathogens and thereby exert a direct negative effect on herbivores (Vega et al., 2009; Ownley et al., 2010). Interestingly, the lifestyle as an insect parasite might be the ancestral state of many plant endophytic fungi, including type I endophytes (Rodriguez et al., 2009). In fact, parasitic fungi can often infect a wide spectrum of hosts including plants, animals, and other fungi. For example, the ubiquitous fungus *Beauveria bassiana* (Ascomycota) has been found as an endophyte in banana, corn, cotton, potato, tomato, common cocklebur, opium poppy, coffee, and cocoa (for references, see Gurulingappa et al., 2010) but is also known to occur naturally in hundreds of insect hosts (Vega et al., 2009; Ownley et al., 2010). Because the effects on the various hosts can be highly different, a surprisingly high number of fungi cause no symptoms in plants but act as severe pathogens when infecting an insect host (Ownley et al., 2010). A likely scenario appears to be that the fungus uses the plant as a vector, that is, to enhance its transmission rate among different insect hosts.

Whereas the direct effect of entomopathogenic fungi on herbivores happens outside the plant, a third, indirect mechanism results from effects of endophytes on the expression levels of host resistance genes. An infection with the root endophytic fungus *A. strictum* enhanced resistance of broad bean (*V. faba*) to the generalist herbivore *H. armigera*. Because the fungus could not be reisolated from the leaves, the authors suggested that it caused a systemic resistance effect and that the antiherbivore effect did not depend on a direct contact between fungus and herbivore (Jaber and Vidal, 2010). It appears to be likely that in most of these cases, the enhanced resistance, at least in part, results from changed expression patterns of resistance genes of the host plant (Ownley et al., 2010). Indeed, the colonization of

plants with fungal endophytes can increase the production of endogenous JA in the host (Ren and Dai, 2012).

Finally, the presence of endophytes enhances the genetic and, consequently, biochemical diversity of the leaf tissue, which should represent per se a beneficial effect in evolutionary terms, because this increased diversity can make the adaptation of herbivores to certain plants more difficult (Schulz and Boyle, 2005; Albrectsen et al., 2010).

114.4 THE POTENTIAL OF ENDOPHYTES IN BIOCONTROL

Fungal entomopathogens and mycoparasites are used as tools in biocontrol. Many commercial "biocontrol" entomopathogens were isolated originally from symptomless plant tissues (Ownley et al., 2010) and thus can be considered as endophytes, at least during a part of their life cycle. Likewise, the resistance-enhancing effects of multiple fungal and bacterial endophytes have recently attracted much research interest due to their potential in biocontrol (Table 114.1; see de Bruijn, 2013). Colonization by these endophytes comes at no measureable cost to the host and can significantly enhance the resistance to pests and pathogens. Recent reports on a resistance-enhancing effect of the treatment of crops with endophytes comprise *Bacillus* sp. enhancing resistance in cacao to black pod rot caused by the oomycete *Phytophthora capsici* (Melnick et al., 2008), *Trichoderma* fungal endophytes protecting cacao from the witches' broom pathogen *Moniliophthora perniciosa* (De Souza et al., 2008), the protection of tomato against pathogenic *Verticillium* strains by the colonization of a nonvirulent strain of the same species (Shittu et al., 2009), and the protection of rice colonized by an endophytic *Azospirillum* strain against the bacterial pathogen *Xanthomonas oryzae* and the rice blast fungus *M. oryzae* (Yasuda et al., 2009). Likewise, treating harvested papaya fruits with the endophytic bacterium *P. putida* MGY2 enhanced their resistance to anthracnose caused by *Colletotrichum gloeosporioides* (Shi et al., 2011), whereas a *Pseudomonas* strain enhanced resistance of potato to soft rot disease caused by the necrotrophic pathogen *Pectobacterium atrospeticum* (Pavlo et al., 2011). In consequence, endophytes that colonize the roots or aerial parts of crop plants are increasingly considered as highly promising means of environmentally friendly crop protection.

However, much more research will be required before certain strains of endophytes can be considered reliable biocontrol agents for certain crops (see de Bruijn, 2013). First, knowledge on the ecology of the entomopathogens is limited (Rodriguez et al., 2009; Vega et al., 2009; Roy et al., 2010), and it remains to be proven whether their negative effects on their insect hosts can positively feed back to plant fitness. Second, as discussed previously, many endophytes realize their resistance-enhancing effect via their influence on the expression of host resistance genes. Therefore, it depends on the detailed type of interaction of a given endophyte with the immune system of its host to which pathogen the host will become resistant. In this respect, endophytes with direct effects on the plant enemies such as entomopathogens, mycoparasites, and endophytes that produce hydrolytic enzymes such as chitinases and ß-glucanases or secondary compounds might be more promising than endophytes that mainly enhance host resistance via interactions with the host immune system. Third, although costs of the colonization by endophytes are rarely reported, these costs are likely to exist: in the end, the endophyte receives all its nutrients from the host plant. This diversion of nutrients to the endophyte might not cause visible effects under high-nutrient conditions, but it will significantly affect plant growth when a nutrient becomes limiting that is shared among host and endophyte. PGPRs with their positive effects on plant nutrition and growth might represent the most promising candidates to circumvent or at least minimize this problem.

Finally, many endophytes can shift from an endophytic to a pathogenic lifestyle. Both lifestyles (endophyte and necrotrophic pathogen) have been reported for *Alternaria alternata* (Huang et al. 2009; Yu et al. 2011; Park et al. 2012), *Colletotrichum acutatum* (Than et al. 2008; Glenn and Bodri, 2012), *C. gloeosporioides* (Promputtha et al. 2007; Than et al. 2008; Yan et al. 2011; Choi et al. 2012), *Colletotrichum musae* (Photita et al. 2005; Promputtha et al. 2007), *Exserohilum rostratum* (Lin et al. 2011; Loro et al. 2012), and *Glomerella cingulata* (Sette et al. 2006; Kwon et al. 2012). Whether some of these reports might refer to cryptic species comprising distinct strains with different ecologies, both lifestyles have been reported for the same strain in the case of *Deightoniella torulosa* (Photita et al. 2004), *Leptosphaeria maculans* (Junker et al. 2012), *Diplodia mutila* (Álvarez-Loayza et al., 2011), and *Lasiodiplodia theobromae* (Sakalidis et al. 2011). Climatic conditions determined whether *Discula quercina* behaved as a pathogen or an endophyte in oak (Moricca and Ragazzi 2011) and high light intensity caused *D. mutila*, which represents a common symptomless endophyte of the tropical palm (*Iriartea deltoidea*), to switch to pathogenicity and cause necrosis (Alvarez-Loayza et al. 2011). A recent phylogenetic analysis demonstrates that this situation is likely to represent the rule rather than the exception (Delaye et al., 2013).

As exemplified elsewhere (Partida-Martinez and Heil, 2011), the outcome of the colonization of a plant with a certain endophyte is highly conditional and depends on the genotype of the endophyte and of the host as well as on abiotic conditions. Thus, the same endophyte might, or might not, cause resistance against a certain pathogen in a certain host plant species depending on inoculum density, host cultivar, and abiotic conditions (Ardanov et al., 2012).

In conclusion, we will need much more detailed understanding of the mechanisms via which endophytes affect the resistance phenotype of their host and of the conditions under which colonization by them harms rather than helps the host plant before endophytes can be considered a safe tool in biocontrol. However, based on the existing knowledge, one might predict that endophytes with direct effects on the enemies of the plant appear in general to be the more promising candidates than those that "only" trigger host resistance genes.

114.5 ENDOPHYTE OR PATHOGEN: WHO CONTROLS?

As defined previously, endophytes are microorganisms that colonize the living tissue of their host without causing symptoms of disease. However, endophytes do infect their host and extract nutrients. Why are endophytes, then, not perceived as pathogens and cause disease? Indeed, several fungi do shift along the mutualist–parasite continuum and behave as pathogens under certain circumstances. Can endophytes escape from the host pathogen perception machinery, do they actively suppress host resistance (just like many successful pathogens do), or are they involved in specific signaling processes to chemically identify themselves to the host as mutualists, as it has been described for mycorrhiza and rhizobia?

First, we have to realize that many endophytes indeed are perceived as pathogens and readily induce host resistance responses, an effect that appears to be the general mechanism that underlies their "indirect" effects. Due to the operational nature of the definition of "endophyte", any microorganism that actually does not cause symptoms of disease qualifies as an endophyte, although the lack of visible disease symptoms might be the outcome of a delicate balance between host resistance responses maintaining the growth rate of the microorganisms at a tolerable magnitude and the microorganisms suppressing host resistance just enough to enable colonization.

Distinguishing that strictly between symptomless endophytes and "not very virulent pathogens" is somewhat artificial because, for the plant, the effect on its net fitness is way more important than whether or not the colonization by the microorganisms locally causes symptoms of disease. Even pathogens can positively affect the net fitness of their host, for example, in the case of the ergot fungus (*Claviceps purpurea*), which produces alkaloids that cause severe toxicity in mammals (Wäli et al., 2013). The frequency of ergot-infected grasses was much higher in sheep pastures than in surrounding ungrazed areas, which makes a positive selection by grazing on the preservation of this infection highly likely (Wäli et al., 2013). In summary, the net effects of the colonization of a plant by a specific endophytic microorganism are not defined by whether or not this microorganism causes visible symptoms of disease. Rather, it is the net sum of all its (often conditional) positive effects (growth promotion, resistance enhancement, etc.) and of all its negative effects (which invariably comprise the usage of nutrients but also might include the suppression of certain defense signaling pathways) that decides whether the colonization of a plant by a microorganism can be considered a mutualism, parasitism, or commensalism under a defined set of environmental conditions. However, it remains an intriguing phenomenon that some microorganisms do not cause any visible resistance response by the host, whereas others cause disease, and future comparative studies will have to identify the factors that decide where on the "fine line" (Junker et al., 2012) from parasite to mutualist a given endophyte–host combination will be most likely to be found.

114.6 SUMMARY AND OUTLOOK

Bacterial and fungal endophytes have been isolated from all parts of all plants that have been searched for them so far and thus can be considered to be ubiquitous. Although per definition they produce no symptoms of disease, endophytes affect multiple phenotypic traits of their host plants, being the most commonly reported effects: (i) resistance to herbivores caused by type I fungal endophytes, (ii) resistance to pathogens by type II fungal endophytes and (iii) growth promotion, and (iv) resistance to pathogens caused by bacterial endophytes. These effects are achieved via multiple different mechanisms which to different degrees depend on the genotype of both host and endophyte. Type II endophytes usually grow at low densities in their host, and thus, no single strain reaches dominance (Lodge et al., 1996; Porras-Alfaro and Bayman, 2011). By contrast, type I endophytes are transmitted vertically, and usually, a single strain colonizes and dominates a given host. Endophytes with their multiple growth-promoting and resistance-enhancing effects are likely to affect multiple plant traits in natural ecosystems and bear potential for a regular use in biocontrol. However, generalizations are still difficult. We lack reliable lists of endophytes that are naturally colonizing a specific host. The complete contribution that endophytes make to the phenotype of their host as we see it in a given environment has never been evaluated. Petri dish assays findings, for example, antibacterial or antifungal effects, do not always predict the behavior of the endophyte *in planta*, let alone its net effect on the phenotype and, ultimately, the fitness of the host. Finally, many endophytes can shift to pathogenic behavior under certain environmental conditions. In summary, inoculating crops with specific endophytes are certainly a highly interesting perspective, but more research will be required before we understand the true contribution of endophytes to

the phenotypes of plants in nature and before endophytes can be used as reliable tools in biocontrol.

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