Plant Developmental Biology – Biotechnological Perspectives

Volume 1

Plant Developmental Biology - Biotechnological Perspectives: Volume 1

Eng-Chong Pua • Michael R. Davey
Editors

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Editors
Prof. Dr. Eng-Chong Pua
New Era College
Jalan Bukit
43000 Kajang
Selangor
Malaysia
eng.chong.pua@gmail.com

Dr. Michael R. Davey
Plant and Crop Sciences Division
School of Biosciences
University of Nottingham
Sutton Bonington Campus
Loughborough LE12 5RD
UK
mike.davey@nottingham.ac.uk

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Preface

Many exciting discoveries in recent decades have contributed new knowledge to our understanding of the mechanisms that regulate various stages of plant growth and development. Such information, coupled with advances in cell and molecular biology, is fundamental to crop improvement using biotechnological approaches.

Two volumes constitute the present work. The first, comprising 22 chapters, commences with introductions relating to gene regulatory models for plant development and crop improvement, particularly the use of Arabidopsis as a model plant. These chapters are followed by specific topics that focus on different developmental aspects associated with vegetative and reproductive phases of the life cycle of a plant. Six chapters discuss vegetative growth and development. Their contents consider topics such as shoot branching, bud dormancy and growth, the development of roots, nodules and tubers, and senescence. The reproductive phase of plant development is in 14 chapters that present topics such as floral organ initiation and the regulation of flowering, the development of male and female gametes, pollen germination and tube growth, fertilization, fruit development and ripening, seed development, dormancy, germination, and apomixis. Male sterility and self-incompatibility are also discussed.

Volume 2 has 20 chapters, three of which review recent advances in somatic embryogenesis, microspore embryogenesis and somaclonal variation. Seven of the chapters target plant processes and their regulation, including photosynthate partitioning, seed maturation and seed storage protein biosynthesis, the production and regulation of fatty acids, vitamins, alkaloids and flower pigments, and flower scent. This second book also contains four chapters on hormonal and environmental signaling (amino compounds-containing lipids, auxin, cytokinin, and light) in the regulation of plant development; other topics encompass the molecular genetics of developmental regulation, including RNA silencing, DNA methylation, epigenetics, activation tagging, homologous recombination, and the engineering of synthetic promoters.

These books will serve as key references for advanced students and researchers involved in a range of plant-orientated disciplines, including genetics, cell and molecular biology, functional genomics, and biotechnology.

August 2009 *E. C. Pua and M. R. Davey*

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Contributors

E. Albertini Department of Applied Biology, University of Perugia, Borgo XX Giugno 4, Perugia 06121, Italy, emidio.albertini@unipg.it

M. Aldana Instituto de Ciencias Físicas, Universidad Nacional Autónoma de México, Campus Cuernavaca, Morelos 62210, Mexico

E.R. Alvarez-Buylla Departamento de Ecología Funcional, Instituto de Ecología, Universidad Nacional Autónoma de México, 3er Circuito Exterior Junto a Jardín Botánico, CU, Coyoacán, Distrito Federal 04510, Mexico, eabuylla@gmail.com, ealvarez@ ecologia.unam.mx

G.C. Angenent Department of Plant Cell Biology, Radboud University Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands Plant Research International, Bioscience, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands, gerco.angenent@wur.nl

G. Barcaccia Genetics Laboratory, Department of Agronomy and Crop Science, University of Padova, Viale dell'Università 16, Legnaro (Padova) 35020, Italy

J.M. Barrero CSIRO Plant Industry, P.O. Box 1600, Canberra, ACT 2601, Australia, jose.barrero@csiro.au

S. Baud Institut Jean-Pierre Bourgin (IJPB), Seed Biology Laboratory, UMR 204 INRA/AgroParisTech, 78026 Versailles Cedex, France

T. Beeckman Department of Plant Systems Biology, VIB, Technologiepark 927, 9052 Ghent, Belgium Department of Molecular Genetics, Ghent University, Technologiepark 927, 9052 Ghent, Belgium, tom.beeckman@psb.vib-ugent.be

M. Bemer Department of Plant Cell Biology, Radboud University Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands

M. Benítez Departamento de Ecología Funcional, Instituto de Ecología, Universidad Nacional Autónoma de México, 3er Circuito Exterior Junto a Jardín Botánico, CU, Coyoacán, Distrito Federal 04510, Mexico

M. Bouzayen Université de Toulouse, INP-ENSA Toulouse, Génomique et Biotechnologie des Fruits, Avenue de l'Agrobiopole, BP 32607, 31326 Castanet-Tolosan, France INRA, Génomique et Biotechnologie des Fruits, Chemin de Borde Rouge, 31326 Castanet-Tolosan, France, bouzayen@ensat.fr

Á. Chaos Departamento de Ecología Funcional, Instituto de Ecología, Universidad Nacional Autónoma de México, 3er Circuito Exterior Junto a Jardín Botánico, CU, Coyoacán, Distrito Federal 04510, Mexico

C.D. Chase Horticultural Sciences Department, University of Florida, Gainesville, FL 32611, USA, cdchase@ufl.edu

I. Debeaujon Institut Jean-Pierre Bourgin (IJPB), Seed Biology Laboratory, UMR 204 INRA/AgroParisTech, 78026 Versailles Cedex, France

B. de Rybel Department of Plant Systems Biology, VIB, Technologiepark 927, 9052 Ghent, Belgium Department of Molecular Genetics, Ghent University, Technologiepark 927, 9052 Ghent, Belgium

K. D'haeseleer Department of Plant Systems Biology, Flanders Institute for Biotechnology (VIB), Technologiepark 927, 9052 Ghent, Belgium Department of Plant Biotechnology and Genetics, Ghent University, Technologiepark 927, 9052 Ghent, Belgium

C. Dubos Institut Jean-Pierre Bourgin (IJPB), Seed Biology Laboratory, UMR 204 INRA/AgroParisTech, 78026 Versailles Cedex, France

B. Dubreucq Institut Jean-Pierre Bourgin (IJPB), Seed Biology Laboratory, UMR 204 INRA/AgroParisTech, 78026 Versailles Cedex, France

A. El-Kereamy Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada N1G 2W1

G.J. Escalera-Santos Departamento de Ecología Funcional, Instituto de Ecología, Universidad Nacional Autónoma de México, 3er Circuito Exterior Junto a Jardín Botánico, CU, Coyoacán, Distrito Federa l04510, Mexico

H. Ezura Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8572, Japan, ezura@gene.tsukuba.ac.jp

M. Falcinelli Department of Applied Biology, University of Perugia, Borgo XX Giugno 74, Perugia 06121, Italy

S. Gan 134A Plant Science, Department of Horticulture, Cornell University, Ithaca, NY 14853-5904, USA, sg288@cornell.edu

S. Goormachtig Department of Plant Systems Biology, Flanders Institute for Biotechnology (VIB), Technologiepark 927, 9052 Ghent, Belgium Department of Plant Biotechnology and Genetics, Ghent University, Technologiepark 927, 9052 Ghent, Belgium

F. Gubler CSIRO Plant Industry, P.O. Box 1600, Canberra, ACT 2601, Australia

D.R. Guevara Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada N1G 2W1

E. Heberle-Bors Department of Plant Molecular Biology, Max F. Perutz Laboratories, Vienna 1030, Austria

K. Hiwasa-Tanase Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8572, Japan

M. Holsters Department of Plant Systems Biology, Flanders Institute for Biotechnology (VIB), Technologiepark 927, 9052 Ghent, Belgium Department of Plant Biotechnology and Genetics, Ghent University, Technologiepark 927, 9052 Ghent, Belgium, marcelle.holsters@psb.vib-ugent.be

D. Horvath United States Department of Agriculture-Agricultural Research Station, Biosciences Research Laboratory, P.O. Box 5674, State University Station, Fargo, ND 58105-5674, USA, horvathd@fargo.ars.usda.gov

A. Isogai Graduate School of Biological Sciences, Nara Institute of Science and Technology, Nara 630-0192, Japan

J. Jacobsen CSIRO Plant Industry, P.O. Box 1600, Canberra, ACT 2601, Australia

L. Jansen Department of Plant Systems Biology, VIB, Technologiepark 927, 9052 Ghent, Belgium Department of Molecular Genetics, Ghent University, Technologiepark 927, 9052 Ghent, Belgium

P. Kaothien-Nakayama, Graduate School of Biological Sciences, Nara Institute of Science and Technology, Nara 630-0192, Japan

Y. Komeda The University of Tokyo, Graduate School of Science, Department of Biological Sciences, Laboratory of Plant Science, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, komeda-y@biol.s.u-tokyo.ac.jp

A. Latché Université de Toulouse, INP-ENSA Toulouse, Génomique et Biotechnologie des Fruits, Avenue de l'Agrobiopole, BP 32607, 31326 Castanet-Tolosan, France INRA, Génomique et Biotechnologie des Fruits, Chemin de Borde Rouge, 31326 Castanet-Tolosan, France

L. Lepiniec Institut Jean-Pierre Bourgin (IJPB), Seed Biology Laboratory, UMR 204 INRA/AgroParisTech, 78026 Versailles Cedex, France, lepiniec@versailles.inra.fr

C.M. Liu Center for Signal Transduction & Metabolomics (C-STM), Institute of Botany, Chinese Academy of Sciences, Nanxincun 20, Fragrant Hill, Beijing 100093, China, cmliu@ibcas.ac.cn

A. Marion-Poll Institut Jean-Pierre Bourgin (IJPB), Seed Biology Laboratory, UMR 204 INRA/AgroParisTech, 78026 Versailles Cedex, France

R.C. Martin USDA-ARS, National Forage Seed Production Research Center, Corvallis, OR 97331, USA

A. Mazzucato Department of Agrobiology and Agrochemistry, University of Tuscia, Via S.C. de Lellis snc, Viterbo 01100, Italy

M. Miquel Institut Jean-Pierre Bourgin (IJPB), Seed Biology Laboratory, UMR 204 INRA/AgroParisTech, 78026 Versailles Cedex, France

W.L. Morris Plant Products and Food Quality, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK

P. Nath Plant Gene Expression Laboratory, National Botanical Research Institute, Rana Pratap Marg, Lucknow 226 001, India

H. Nonogaki Department of Horticulture, Oregon State University, Corvallis, OR 97331, USA, hiro.nonogaki@oregonstate.edu

H. North Institut Jean-Pierre Bourgin (IJPB), Seed Biology Laboratory, UMR 204 INRA/AgroParisTech, 78026 Versailles Cedex, France

P. Padilla-Longoria Instituto de Investigaciones en Matemáticas Aplicadas y en Sistemas, Universidad Nacional Autónoma de México, Distrito Federal 04510, Mexico

J.C. Pech Université de Toulouse, INP-ENSA Toulouse, Génomique et Biotechnologie des Fruits, Avenue de l'Agrobiopole, BP 32607, 31326 Castanet-Tolosan, France INRA, Génomique et Biotechnologie des Fruits, Chemin de Borde Rouge, 31326 Castanet-Tolosan, France

W.E. Pluskota Department of Plant Physiology and Biotechnology, University of Warmia and Mazury, Oczapowski 1A, 10-718 Olsztyn, Poland

A. Ribarits Department of Plant Molecular Biology, Max F. Perutz Laboratories, Vienna 1030, Austria

C. Rochat Institut Jean-Pierre Bourgin (IJPB), Seed Biology Laboratory, UMR 204 INRA/AgroParisTech, 78026 Versailles Cedex, France

S.J. Rothstein Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada N1G 2W1

J.-M. Routaboul Institut Jean-Pierre Bourgin (IJPB), Seed Biology Laboratory, UMR 204 INRA/AgroParisTech, 78026 Versailles Cedex, France

S.D. Russell Department of Botany and Microbiology, University of Oklahoma, Norman, OK 73019, USA, srussell@ou.edu

N. Sánchez-León National Laboratory of Genomics for Biodiversity, Cinvestav Campus Guanajuato, Km 9.6 Libramiento Norte Carretera Irapuato-Leon, CP36500 Irapuato Guanajato, Mexico

T.F. Sharbel Apomixis Research Group, Department of Cytogenetics, Institut für Pflanzengenetik und Kulturpflanzenforshung, Corrensstrasse 3, 06466 Gatersleben, Germany

D.-Q. Shi The CAS Key Laboratory of Molecular and Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China

S. Takayama Graduate School of Biological Sciences, Nara Institute of Science and Technology, Nara 630-0192, Japan, takayama@bs.naist.jp

M.A. Taylor Plant Products and Food Quality, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK, mark.taylor@scri.ac.uk

D. Twell Department of Biology, University of Leicester, Leicester LE1 7RH, UK, twe@ le.ac.uk

V. Vassileva Academik Metodi Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Academik Georgi Bonchev Street, Building 21, Sofia 1113, Bulgaria

R. Verduzco-Vázquez Facultad de Ciencias, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos 62210, Mexico

J.-P. Vielle-Calzada National Laboratory of Genomics for Biodiversity, Cinvestav Campus Guanajuato, Km 9.6 Libramiento Norte Carretera Irapuato-Leon, CP36500 Irapuato Guanajato, Mexico, vielle@ira.cinvestav.mx

M.W.F. Yaish Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada N1G 2W1, myaish@uoguelph.ca

H. Yamashita Kyoto Prefectural University, Faculty of Life and Environmental Sciences, Laboratory of Plant Molecular Biology, Shimogamo-nakaragi-cho, Sakyo-ku, Kyoto 606-8522, Japan

W.-C. Yang The CAS Key Laboratory of Molecular and Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China, wcyang@genetics.ac.cn

C. Zhou 134A Plant Science, Department of Horticulture, Cornell University, Ithaca, NY 14853-5904, USA College of Life Sciences, Hebei Normal University, Shijiazhuang, Hebei 050016, China

Part I
Models for Plant Development

Chapter 1
Gene Regulatory Models for Plant Development and Evolution

E.R. Alvarez-Buylla, M. Benítez, M. Aldana, G.J. Escalera-Santos, Á. Chaos, P. Padilla-Longoria, and R. Verduzco-Vázquez

1.1 Introduction: the Need for Mathematical Models to Understand Plant Development

During development, complex interactions amongst genetic and non-genetic elements give rise to robust spatiotemporal patterns. Moreover, an important feature of biological systems is the nontrivial flow of information at several scales. When we consider the scale determined by the cell, we observe that it integrates information coming from gene regulatory networks (GRNs), biochemical pathways, and other microscopic processes. If we consider larger scales, then intercellular communication, mechanical and geometric effects (such as growth, shape, and size), and environmental influences have to be taken into account. This is why understanding how patterns arise during development requires the use of formal dynamical models able to follow the concerted action of so many elements at different spatiotemporal scales.

E.R. Alvarez-Buylla, M. Benítez, M. Aldana, G.J. Escalera-Santos, Á. Chaos, P. Padilla-Longoria, and R. Verduzco-Vázquez
C3, Centro de Ciencias de la Complejidad, Cd. Universitaria, UNAM, México, D. F., México

E.R. Alvarez-Buylla, M. Benítez, G.J. Escalera-Santos, and Á. Chaos
Departamento de Ecología Funcional, Instituto de Ecología, Universidad Nacional Autónoma de México, 3er Circuito Exterior Junto a Jardín Botánico, 04510 Distrito Federal, Coyoacán, CU, Mexico
e-mail: eabuylla@gmail.com, ealvarez@ecologia.unam.mx

M. Aldana
Instituto de Ciencias Físicas, Universidad Nacional Autónoma de México, Campus Cuernavaca, Morelos, 62210, Mexico

P. Padilla-Longoria
Instituto de Investigaciones en Matemáticas Aplicadas y en Sistemas, Universidad Nacional Autónoma de México, 04510, Distrito Federal, Mexico

R. Verduzco-Vázquez
Facultad de Ciencias, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos, 62210, Mexico

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The fact that biological entities and scales often interact nonlinearly makes mathematical modeling of biological systems, and in particular of gene regulatory networks, a nontrivial problem. From the mathematical point of view, the incorporation of all these interactions can be taken into account only by implementing hybrid models, that is, by incorporating both discrete and continuous elements, as well as deterministic and stochastic frameworks. In fact, depending on the specific space-time scale at which a process is being observed, it might appear discrete or continuous, deterministic or random. For instance, the levels of gene expression might be taken as discrete (the gene is "on" or "off") when seen at rough space-time scales, but when observed with a finer gauge, these levels appear as continuously varying.

Mathematical models of GRNs provide an integrative tool, a systematic way of putting together and interpreting experimental information about the concerted action of gene activity. They also offer new insights on the mechanisms underlying biological processes, in particular developmental ones, as well as a means to make informed predictions on the behavior of such complex systems.

1.2 Dynamic GRN Models

Today, one of the most important challenges in systems biology is to relate the gene expression patterns of an organism with its observed phenotypic traits. Since these patterns result from the mutual activation and inhibition of all the genes in the genome in a coordinated way, the above problem is equivalent to relating the dynamical properties of the underlying genetic network with the organism's phenotype (Hasty et al. 2001; Levine and Tjian 2003). In order to achieve this goal, one must decide first how to model the dynamics of the genetic network.

Amongst the several theoretical approaches that have been proposed to model the genetic dynamics, two stand out, namely, the *continuous* and the *discrete* (Smolen et al. 2000; Bower and Bolouri 2001). The continuous approach is based on systems of coupled nonlinear differential equations that describe the temporal evolution of the concentration of the chemicals involved in the gene regulation processes (proteins, enzymes, transcription factors, metabolites). This description is particularly suitable when the systems under consideration consist of a small number of components (Arkin et al. 1998; Vilar et al. 2003). However, large-scale genome analysis has revealed that the coordinated expression of dozens, or even hundreds of genes is required for many cellular processes to occur, such as cell division or cell differentiation (Whitfield et al. 1992; Rustici et al. 2004). For such processes, the continuous approach becomes intractable due to the great number of components and equations involved.

The discrete approach to model the dynamics of genetic networks was first introduced by Kauffman to describe, in a qualitative way, the processes of gene regulation and cell differentiation (Kauffman 1969). This approach focuses on the state of expression of the genes, rather than on the concentration of their products.

Thus, the level of expression of a given gene is represented by a discrete variable g that usually takes the values g=0 if the gene is not expressed, and g=1 if the gene is fully expressed. The genome is considered then as a set of N discrete variables, $g_1, g_2, \ldots, g_N$, their values changing in time according to:

$$g_n(t+1) = F_n\left[g_{n_1}(t), g_{n_2}(t), \ldots, g_{n_{k_n}}(t)\right] \tag{1.1}$$

In this equation, ($g_{n_1}, g_{n_2}, \ldots, g_{n_{k_n}}$) are the k_n regulators of the gene g_n, and F_n is a discrete function (also known as a logical rule) constructed according to the nature of the regulators. The advantage of the discrete model is that it can incorporate a much larger number of components than the continuous models. Furthermore, recent work shows evidence that, in spite of the simplicity of the discrete approach, it is able to reproduce the gene expression patterns observed in several organisms (Huang and Ingber 2000; Mendoza and Alvarez-Buylla 2000; Albert and Othmer 2002; Espinosa-Soto et al. 2004; Davidich and Bornholdt 2008). This evidence has been obtained for relatively small genetic networks for which both the regulators and the logical rules are known for each gene.

Accumulated data on molecular genetics and current high-throughput technology (see next section) have made available a great amount of data regarding GRNs, yet information for all the regulators and logical rules in entire genomes is not available yet for any organism. Nonetheless, it is important to emphasize that, for the small genetic modules or sub-networks that have been thoroughly documented experimentally, the discrete approach gives accurate predictions.

Arguably, one of the most important results of the discrete model is the existence of *dynamical attractors*. Starting out from an initial state [$g_1(0), g_2(0), \ldots, g_N(0)$] in which some genes are active and some others inactive, Eq. (1.1) generates dynamics in which each gene goes through a transient series of active/inactive states until the whole network enters into a periodic pattern of expression (Fig. 1.1). Some genes reach a constant value that does not change in time anymore, whereas some others keep "blinking" in a periodic way. This periodic state of expression of the entire network is the dynamical attractor. The set of all the possible initial states that after a transient time fall into the same attractor is called the *basin of attraction* of that attractor. Each attractor is uniquely identified by its set of active genes. In other words, particular sets of genes are expressed in different attractors, and this is precisely the characteristic that identifies the different functional states of the cell. For this reason, Kauffman formulated the hypothesis—confirmed experimentally—that the dynamical attractors of the genetic network correspond to the different cell types or cell fates observed in the organism.

Since the level of expression of each gene is discretized into a finite number of values, the total number, Ω, of dynamical states in which the network can be found is also finite, and is given by $\Omega = \prod_{n=1}^{N} m_n$, where m_n is the number of discrete values that g_n can acquire. Under Eq. (1.1), the dynamical space of the network (i.e., the possible Ω states) is partitioned into disjoint sets consisting of the attractors and their corresponding basins of attraction (Fig. 1.1). This structure of the dynamical

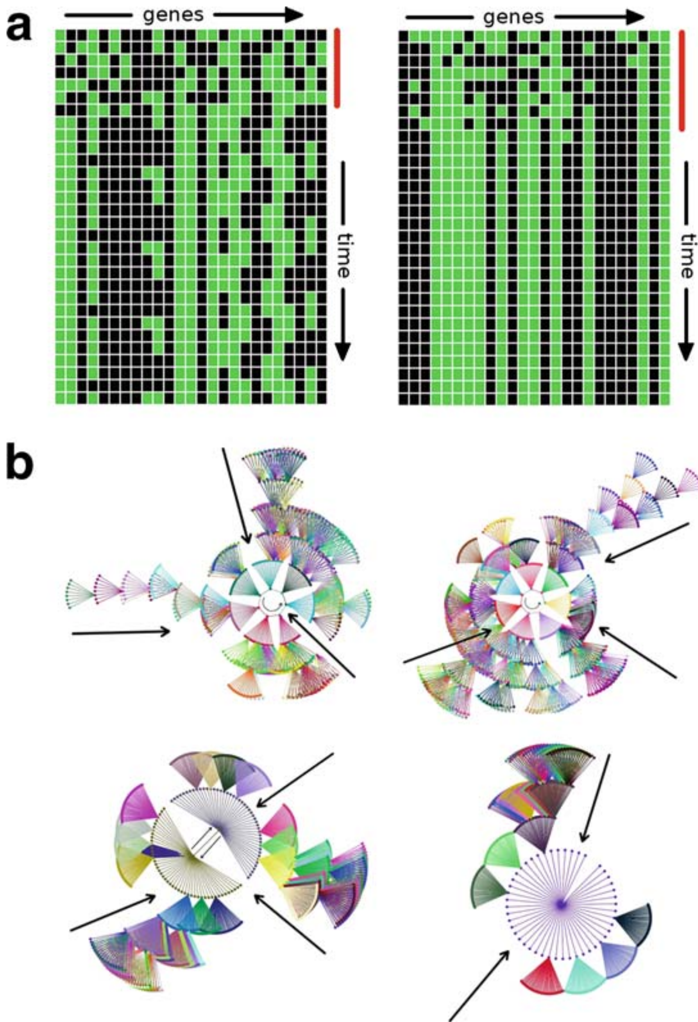

Fig. 1.1 Attractors and attractor basin in a GRN. **(a)** Visual representation of the dynamical attractors of a genetic network. Each square represents a gene, in *gray* if it is expressed, and in *black* if it is not. The genes are lined up horizontally so that each row represents the state of expression of the entire genome at a given time. Time flows downward. After a transient time (indicated with a *vertical line*), the whole network reaches a periodic pattern of expression, which is the dynamical attractor. As shown, two different initial states (the *uppermost rows*) can lead to different attractors. The attractor on the *left* has period six, whereas the attractor on the *right* consists of only one state. **(b)** Visual representation of the attractor landscape for a randomly constructed network with $N = 12$ genes. Each *dot* represents a dynamical state of the network (i.e., one of the rows in **a**), and the *lines* represent discrete time steps. Two dots are connected if they are successive states under the dynamics given by Eq. (1.1). The fan-like structures reflect the fact that many states can have the same successor in time (the dynamics are dissipative). The *arrows* indicate the direction of the dynamical flow. In this particular example, the state-space of possible dynamical states organizes into four disjoint sets consisting of the attractors and their respective basins of attraction

space is called the *attractor landscape*, and constitutes a representation of the epigenetic landscape conceived by Waddington (1957) to qualitatively understand the different functional states of the cell. It has been shown recently for several cases that many important phenotypic traits of the organism, such as the cell type or the cell cycle, are encoded in the entire attractor landscape.

1.3 Inference of GRN Topology from Microarray Experiments

GRN architecture inference is the process by means of which information on the regulators is obtained from experimental data. In some cases, network structure has been inferred from thorough data of molecular genetics experiments, enabling novel insights and predictions for particular developmental systems (Mendoza and Alvarez-Buylla 2000; Albert and Othmer 2002; Espinosa-Soto et al. 2004). Nevertheless, the current available technology enables the generation of large sets of genomic information, commonly acquired from microarray experiments. This experimental technique allows observing the expression pattern of a set of genes at different sample points in time or under different experimental conditions, and has generated a vast data base.

Although powerful, microarray experiments and their data have two difficulties. First, an enormous number of experiments are necessary in order to confidently infer all the logical rules in a given genome. Second, the data obtained are very noisy, which is why uncovering structural or dynamic information is anything but trivial. We briefly introduce some of methods and approaches that have addressed the need of formal frameworks in this area.

Reverse engineering is the process of discovering the functional principles of a device, object, or system through analysis of its structure, function, or operation. In the context of GRNs, it constitutes the process of network structure inference from the analysis of experimental data on gene expression under diverse conditions, often derived from microarray experiments. Despite the particular method to be used to analyze microarray data, the overall goal of GRN reverse engineering is to find mathematical evidence supporting the proposition of an interaction between the nodes of the network.

Two main classes of methods have been proposed to infer GRN architectures via reverse engineering methods. The first class relies on probability theory, and its objective is to find the most probable network architecture given a genetic expression pattern, or to quantify the existent correlation between pairs of genes. Bayesian networks, both traditional and their dynamic variant, fall into the first approach, while mutual information methods fall into the second one. The second class of methods is based on continuous analysis. It involves ordinary differential equations (ODEs), and is supported by the theory and methods from stability analysis of dynamic systems.

1.3.1 Bayesian Networks

A Bayesian network is an acyclic graph of a joint probability distribution where the nodes are the random variables, and the directed edges are causal influences. Bayesian network models have proven to be useful to infer a GRN structure (Imoto et al. 2002). However, one of the major drawbacks of traditional Bayesian models is that, by definition, cycles cannot be found, and cycles or feedback loops constitute a very important feature of biological GRNs. However, dynamical Bayesian network models (Kim et al. 2003) allow both the inclusion of cycles and the representation of a different temporal behavior for each gene of the network, and offer a promising alternative for reverse engineering of GRNs.

1.3.2 Mutual Information

Mutual information is a technique that allows inferring GRN architecture with a more general criterion than that of the more common statistical methods, which focus mainly on linear correlations, as it enables consideration of any functional relationship (see review in Steuer et al. 2002). Despite the advantage of being rooted in a well-known probabilistic framework, these methods are computationally intensive, due to the high amount of nodes, and the estimation of the unknown temporal delays for each node, which has to be approximated, thus limiting the possibility of studying GRNs composed of a large number of nodes.

1.3.3 Continuous Analysis Models

These methods consider a network of n genes as a system of ODEs where the change in the level of expression of gene i is denoted as (x_i), and its dynamic is described as:

$$\frac{\mathrm{d}x_i}{\mathrm{d}t} = f_i(x_1, x_2, \ldots, x_n) \text{ for } i = (1, 2, \ldots, n) \tag{1.2}$$

Thus, the influence that node x_j inflicts on node x_i is obtained by computing the partial derivative of f_i with respect to x_j. Moreover, the sign of each of these partial derivatives determines whether the interaction between a couple of nodes corresponds to up- or downregulation. The set of all so-defined partial derivatives constitutes the Jacobian matrix of the system, and hence, the GRN architecture is obtained as a graphical representation of the signs of the elements of the Jacobian matrix (Aguda and Goryachev 2007). An alternative method to compute the sign of these derivatives consists of perturbing each f_i (see Kholodenko et al. 2002; Sontag et al. 2004; Andrec et al. 2005). In fact, near a steady state, both the perturbation and

the Jacobian matrix methods are theoretically equivalent, and thus yield the same results.

A slightly different approach is suggested by Cho et al. (2006). In this method, each column of a matrix represents the expression profile of gene i at times $t_1, t_2, \ldots, t_k$. This may be regarded as a set of measurements of a random variable x_i. Each time series x_i is then plotted in a phase portrait against each and every x_j such that $j \neq i$. In this case, the direction of the interaction is given by a winding index WI, and the type of interaction by a slope index SI. For instance, considering a two-node network with components x_1 and x_2 whose time-series expression profiles are measured at k even sampling points, SI and WI of x_1 and x_2 are given by:

$$\mathrm{SI}(x_1, x_2) = \frac{1}{k-1} \sum_{i=1}^{k-1} sign \left[\frac{x_2(i+1) - x_2(i)}{x_1(i+1) - x_1(i)} \right] \tag{1.3}$$

$$\mathrm{WI}(x_1, x_2) = \frac{1}{k-2} \sum_{i=1}^{k-2} sign \ [\mathrm{d}(i)] \tag{1.4}$$

where

$$\mathrm{d}(i) = \det \begin{bmatrix} x_1(i) & x_1(i+1) & x_1(i+2) \\ x_2(i) & x_2(i+1) & x_2(i+2) \\ 1 & 1 & 1 \end{bmatrix} \tag{1.5}$$

There are still very few examples of successful applications of these methods of reverse engineering to plant data (cf. review in Alvarez-Buylla et al. 2007). In contrast, dynamic GRN models grounded on detailed molecular genetic plant data have been successful at reproducing observed patterns of gene expression. We, therefore, focus here on such an approach for small sub-networks of plant development.

1.4 GRN Models for Modules of Plant Development

Dynamic network models have been recently used to study plant development, since they are able to capture important aspects of biological complexity. Furthermore, these models integrate empirical evidence, and thus provide a useful tool for novel hypothesis testing by detecting missing or contradictory data, generating predictions, and delimiting future experiments. As mentioned above, most of such models have been based on relatively small and thoroughly described sub-networks associated to a particular developmental process. This has enabled a rather direct interpretation of the model results, and a more profound understanding of certain aspects of development.

1.4.1 *Single-Cell Gene Regulatory Network Models: the Case of Arabidopsis* **Flower Organ Primordial Cell Specification**

In plants, the flower is the most complex and well-studied structure from a developmental perspective. It characterizes angiosperms or flowering plant species, and exhibits a stereotypical conserved structure in the great majority of flowering plant species (Rudall 1987). Early during flower development, the bud or flower meristem is partitioned into four concentric regions, each one comprising the primordia that will eventually form mature floral organs. Floral organs appear from the outermost to the inner part of the plant in the sequence sepals, petals, stamens, and carpels.

There is a great amount of detailed and high-quality data for the molecular interactions that regulate flower development. In fact, on the base of these data, a now classical model of flower development has been proposed, namely, the "ABC" model. This model establishes that the combinatorial activities of genes grouped in three types or functions (*A* type, *B* type, and *C* type) are needed to specify floral organs. *A* genes alone are needed for sepal identity, *A+B* for petal, *B+C* for stamen, and *C* alone for carpel identity (Coen and Meyerowitz 1991).

A GRN Boolean model grounded on experimental data (Mendoza and Alvarez-Buylla 1998; Mendoza et al. 1999; Espinosa-Soto et al. 2004; Chaos et al. 2006) recovers the profiles of gene activation that characterize primordial sepal, petal, stamen, and carpel cells during early *Arabidopsis thaliana* (L.) Heynh. flower development (Fig. 1.2). This was the first published Boolean GRN model that was validated with experimental data, and generated testable predictions. Since then, other systems have been studied with the same approach.

The results of the floral GRN model coincide with the *ABC* model, but also provide a dynamic explanation for the robust attainment of the combinatorial gene activations involved in floral organ determination. In addition, this GRN model enabled hypotheses on the sufficiency and necessity of particular gene regulatory interactions among the ABC and other genes. Computer simulations of this flower GRN also show that its attractors are robust to random perturbations on the logical rules (Espinosa-Soto et al. 2004; Chaos et al. 2006), hinting on an explanation for the evolutionary conservation of flower structure. In conclusion, this model incorporates the key components of the GRN underlying the ABC model, and provides a dynamical explanation for cell type determination in flower buds.

1.4.2 *Spatiotemporal Models of Coupled GRN Dynamics*

The models presented above are useful to explore cell-fate attainment in isolated cells. However, in order to understand the emergence of spatiotemporal cell patterns during development, models that couple such single-cell GRN models in explicit spatial domains are needed.

Most models addressing the origin of cellular patterns consider "toy networks", or dismiss intracellular GRN topology altogether, and provide only mesoscopic

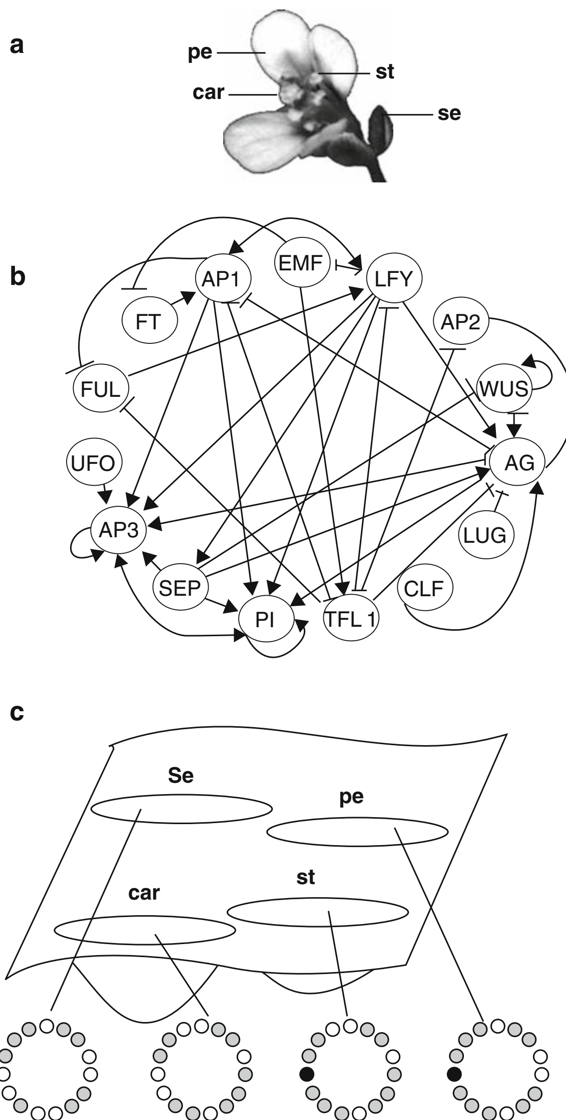

Fig. 1.2 Gene regulatory network underlying cell type determination during early flower development in *Arabidopsis*. **(a)** Mature flower showing the four floral organs: sepals, petals, stamens, and carpels. **(b)** The GRN depicted here underlies the attainment of the primordial cellular identities during flower development. Nodes represent genes, and edges denote regulatory interactions among them (*arrows* correspond to positive regulation, "*flat arrows*" to negative regulation). **(c)** The GRN represented in **b** attains steady states that match the gene activation profiles characteristic of the four primordial cell types. In a schematic landscape, each cell type corresponds to a local minimum, and is associated to a particular GRN configuration (nodes in *white* are "off", those in *gray* are "on", and those in *black* may be in either of the two states)

models of morphogenetic dynamics, while the majority of experimentally grounded GRN models ignore cellular-scale interactions. Therefore, one of the challenges remaining today is to achieve multi-scale models, most likely by the postulation of hybrid models that integrate GRNs in cellular contexts.

During plant development, cells commit to a certain fate according mainly to their position in a region of the plant, rather than in relation to their cellular lineage as is the case in most animal systems (Scheres 2001). Hence, understanding how positional information is generated and maintained comprises a paramount task for developmental biology. GRN dynamics, geometry of the domain, mechanical restrictions, and hormonal and environmental factors all play relevant roles in this process. Below we present two developmental models that partially incorporate some of these aspects.

The GRNs responsible for cell type determination in the leaf and root epidermis of *A. thaliana* have been thoroughly described, and provide an excellent system for addressing the origin of cellular patterning during development. It has been suggested that this network may behave qualitatively as an activator-inhibitor system (Pesch and Hülskamp 2004), which is able to generate stable complex patterns de novo. This has been further explored with the use of a dynamic spatial model (Benítez et al. 2007). In this approach, the authors first used a discrete GRN model, and found that its attractors match two epidermal cell types, corresponding to hair and non-hair cells. Then, the authors simulated a simplified version of the network in a spatial domain, and provided evidence supporting that leaf and root GRNs, although slightly different, are qualitatively equivalent in their dynamics. This study also showed that cell shape may have a relevant role during cell pattern formation in the root epidermis.

Another model that considers a GRN in a spatial domain is that proposed by Jönsson et al. (2005), in which the authors used in vivo gene expression data to simulate a cellularized template incorporating a relatively small GRN. This GRN, which includes the gene *WUSCHEL* (*WUS*), seems to regulate the meristem's size and maintenance, and was modeled with the use of the so-called connectionist model (Mjolsness et al. 1991). By doing this, the authors postulated a mechanism that could underlie the spatial gene expression pattern of *WUSCHEL* (*WUS*) in the A. thaliana shoot apical meristem, and provided a useful experimental and computational platform to improve developmental models. Recently, several platforms helpful for integrating GRNs in a cellularized domain and modeling plant development have been proposed (Holloway and Harrison 2007; Buck-Sorlin et al. 2008; Dupuy et al. 2008). These will be useful for further models of coupled GRNs.

1.4.3 Auxin Transport Is Sufficient to Generate Morphogenetic Shoot and Root Patterns

Morphogene gradients are the key for pattern formation. In plants, auxin is a hormone that provides important positional information during *A. thaliana*

development (Vieten et al. 2007). Recently, some mesoscopic models for auxin-driven pattern formation in the shoot and root have integrated the accumulated experimental evidence, and contributed to the understanding of these systems.

In the growing *A. thaliana* shoot, new leaves and flower primordia emerge at defined positions along the flanks of the shoot apical meristem. Primordia patterning, and therefore phyllotactic arrangements, seem to be determined by auxin peaks that determine site or primordia initiation and activation. On the base of empirical data, Jönsson et al. (2006) suggested a mechanism in which this plant hormone influences its own polarized flux within the shoot apical meristem by directing localization of its own transporters (PIN and AUX proteins). The mathematical model for auxin transport proposed by Jönsson et al. (2006) recovered peaks of auxin concentration at positions where actual new primordia emerge. Their cell-based model revealed that the auxin feedback loop, in which the hormone regulates its own transport, is sufficient to generate the regular spatial patterning of primordia.

The above model is able to generate the complex phyllotactic patterns observed in plants under different parameters. However, in contrast to what has been observed in a great majority of plants, the patterns generated by this model are not stable. We hypothesize that the stability of observed phyllotactic patterns may depend upon the complex GRNs that underlie PIN, AUX, and other protein regulation.

A recent paper (Grieneisen et al. 2007) proposed a computational model that addresses the generation of a robust and information-rich auxin pattern in *A. thaliana* roots. This model assumes certain internal distribution of the PIN auxin transport facilitators, and incorporates diffusion and permeability, as well as the *A. thaliana* root structure. Interestingly, the patterns recovered by this root model are robust to alterations on several parameters, as well as to cell division and expansion. Given the PIN layout in the root, the model is useful to explain the phenotypes of pin loss-of-function mutants, and also accounts for slow changes in root zonation (meristematic and elongation zones) when feedback from cell division and expansion are introduced. According to this work, the auxin pattern depends on a capacitor-like mechanism that may buffer the absence of auxin from the shoot, or auxin leakage and decay.

The study of Grieneisen et al. (2007) is a wonderful example of how a mathematical and computational model can be useful to provide explanations about developmental mechanisms and patterns, and to generate novel hypotheses that can be tested experimentally. Yet, this model stands on the assumption that the auxin transporters maintain a fixed polarized distribution within the cell. Since it has been shown that the transporters' localization is affected by the auxin flux itself, a more general model should incorporate a dynamic mechanism for the mutual regulation of transporter position and auxin flux.

Both models show that transport-dependent auxin gradients constitute a powerful mechanism to generate developmental information, and will certainly provide a solid base to incorporate the genetics of PIN distribution, as well as the role of other components of plant morphogenesis.

1.4.4 Signal Transduction Models

In living organisms, GRNs are often interacting with other sub-networks, or with signaling pathways that act as an input to GRNs. This is particularly clear in plants—being sessile, they respond to environmental challenges by plastic developmental responses. Signaling pathways frequently integrate environmental cues, and are the key for developmental plasticity. These pathways are usually hierarchical, and in a first approximation may be represented as cascade processes. However, these pathways often show complex dynamics, e.g., oscillations and chaos, and crosstalk among them seems to be the rule in plants, which is why dynamical models will certainly be useful for a better understanding of these processes. Some recent models aim at simulating the dynamics of pathways in plants, plastic processes of development, and the coupled dynamics of pathways and GRNs.

Díaz and Alvarez-Buylla (2006) proposed a continuous model that endeavors at studying the signaling pathway associated to the hormone ethylene in *A. thaliana*, as well as the effect of different ethylene concentrations on downstream transcription factors. This model predicts dose-dependent gene activity curves that are congruent with the dose-dependent observed phenotypes, and interestingly, it also leads to the prediction that signaling pathways may filter certain stochastic or rapid fluctuations of hormone concentration.

Also focusing on the dynamics of plant hormones is the model presented by Li et al. (2006). Their model consists of a Boolean network approach that integrates the great amount of experimental findings related to the abscisic acid pathway, and stomata opening and closure dynamics. Such a model is able to predict and test network alterations leading to qualitative changes in the behavior of stomata. Models like this contribute to a better understanding of plant physiology, as well as to the development of better techniques for crop management.

As mentioned above, cell type determination in the *A. thaliana* epidermis has been thoroughly studied, and it has been found that root hair arrangement is plastic with respect to nutrient availability. Savage and Schmidt (2008) present a hypothesis that is congruent with available molecular and physiological data, and that attempts to account for root hair arrangement in a context of developmental plasticity. The mechanism they postulate and simulate relies on a well-known Turing-like patterning mechanism, and remains to be tested experimentally. This is an example of how computational models of plant development may lead to, or eventually support, precise and novel non-intuitive hypotheses.

1.5 The Constructive Role of Stochasticity in GRN and Other Complex Biological Systems

All the above models are deterministic. Historically, noise has been considered as a nuisance, and efforts to control or minimize it have been undertaken. However, the pioneering works of Benzi et al. (1981) and Nicolis (1981, 1982) changed this

perspective, as they showed that noise may play an important role in the appearance of patterns in complex systems.

Benzi et al. (1981) introduced the concept of stochastic resonance (SR) for processes, in which the presence of random fluctuations (noise) amplifies the effects of a weak deterministic signal (Gammaitoni et al. 1998). More recently, the number of studies addressing the interaction of noise and deterministic signals in complex systems has increased (Gang et al. 1993; Pikovsky and Kurths 1997; Russell et al. 1999), and numerous new constructive roles of noise have been acknowledged in diverse natural processes.

Noise is ubiquitous in genetic processes (Rao et al. 2002; Blake et al. 2003; Paulsson 2004; Cai et al. 2006). It can arise from at least two different sources in cells. First, statistical fluctuations from a finite number of molecules make the transcriptional and translational processes intrinsically stochastic (Blake et al. 2003). Second, small variations in temperature and environmental perturbations provide the source for extracellular noise. It appears that GRNs are not only robust to stochastic fluctuations, but in some cases they incorporate noise in a constructive way (Wang et al. 2007). Most studies of this phenomenon have documented noise-enhanced heterogeneity (Rao et al. 2002), which has been proposed as a means for improving sensitivity of intracellular regulation to external signals (Paulsson et al. 2000). A related phenomenon is noise-induced selection of attractors (Kaneko 1998; Kraut et al. 1999), which enables dynamical switching to multistability in systems that are deterministically monostable.

In the context of developmental biology, it has been postulated that cell-fate differentiation can be driven by noise (Huang and Ingber 2007). Therefore, considering noise in dynamic models could be important for analyzing the spatiotemporal sequence with which cell fates are determined during development. For instance, GRNs that underlie cell determination could be viewed as a stochastic dynamical system (Davidson et al. 2002). This approach rescues the original proposal of an epigenetic landscape explored by random perturbations (Waddington 1957) as a metaphor for understanding the dynamical patterns of transitions among different functional states of the cell during development.

1.6 GRN Structure and Evolution

Besides the use of GRNs for understanding the development of extant organisms, such models are useful for exploring hypotheses on organismal evolution. A particularly interesting phenomenon recently reported is that, after the duplication and divergence (through mutations) of a single gene in a network, new attractors can appear (Aldana et al. 2007). Thus, not only can the duplicated and diverged gene acquire a new function (Li and Graur 1991), e.g., a new signaling molecule or structural protein, but also the entire genetic network can develop new phenotypes and functional states. Attractors of GRNs can be interpreted as

characters, cell types, or functions (Huang and Ingber 2000; Espinosa-Soto et al. 2004; Huang et al. 2005), and the number of these affect the possibilities to evolve and adapt. Thus, the emergence of new attractors allows for the possibility of evolving, constituting the raw material upon which natural selection could act.

A second possibility for GRN evolution is the integration of two networks in a way similar to that of an engineer working with capacitors, transistors, and other modular elements. These are combined in various ways to create new devices. This evolutionary process may occur by duplicating the whole network, or by linking two or more independent networks, each one with a particular set of functions. In this way, both networks can continue to yield their original functions, but the interaction between them can originate new functions.

In the different types of GRNs, and thus organismal evolution, particular restrictions operate. Under the second one (network coupling), the resulting network must maintain its original attractors, or at least most of them. If the original attractors were eliminated, it would be very difficult for the organism to survive, because its phenotype would be drastically affected. This mechanism could underlie key evolutionary events—for example, the appearance of eukaryotic cells from the combination of prokaryotic cells, or that of multicellularity from combining unicellular organisms (Margulis and Sagan 1986). Indeed, multicellular organisms are ensembles of complex networks that could have originally underlied single-celled organisms. Therefore, methods enabling the dissection of large networks into subnetworks or modules that have a shared history will be useful to understanding the evolution of large and complex GRNs.

Biological networks are modular and composed of some reiterating subgraphs, but little is known about the evolutionary origin of such components or GRN building blocks. Several contributions on modularity have attempted to understand the connectivity, topology, synchronization, and organization of modules (Ravasz et al. 2002; Kashtan and Alon 2005; Quayle and Bullock 2006; Arenas et al. 2006; Irons and Monk 2007; Wang and Zhang 2007; Siegal et al. 2007). For instance, initial approaches to understanding how networks are locally connected have identified certain types of sub-graphs, called motifs, with a particular connection pattern. The simplest motifs are of three nodes. If the graphs are directed, there are 13 different motifs or connective configurations of three nodes. The relative abundance of these motifs in real networks is not random; different types of networks have different motifs over- or underrepresented (Milo et al. 2004).

Such motif representation patterns may have been selected for, or maybe have resulted as a byproduct of the way networks are assembled—in other words, as a result of neutral processes (Solé and Valverde 2006). Evidence to support both cases exists, and therefore it is still unclear why some motifs are more, or less, common than others. Nevertheless, understanding how biological networks have assembled during the course of evolution is fundamental to comprehend how changes in GRNs map unto evolutionary alterations of developmental processes, and therefore, unto organismal phenotypes.

1.7 Conclusions

Mathematical models grounded on experimental data are now both possible and necessary in order to study the concerted action of the many entities that, at several spatiotemporal scales, intervene during development. Plants are becoming paradigmatic systems to meet the challenge of building these models.

We have reviewed two widely used types of models, discrete and continuous. Nevertheless, the central task of considering the various levels at which developmental processes occur in integrative and realistic models still remains ahead, and it is likely that hybrid models will be needed. So far, dynamical models, and more precisely, gene regulatory network models have provided a powerful means to integrate vast empirical information, test or postulate hypotheses and predictions, and reach novel insights on the nature and evolution of plant developmental processes. Such models will certainly continue to be useful tools as feedback to and from experimental approaches in plant developmental biology.

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Chapter 2
Arabidopsis as Model for Developmental Regulation and Crop Improvement

C.M. Liu

2.1 Introduction

A longstanding argument for using Arabidopsis to carry out basic research is that the ease of studying the model plant speeds up elucidation of the biology of agriculturally important traits, and subsequent transfer of the knowledge to crop improvement. During the last two decades, the Arabidopsis research community has increased very rapidly (Somerville and Koornneef 2002). As the first plant genome sequenced, and the most comprehensive community service in plants available to date, studies using Arabidopsis have covered almost every aspect of plant biology. It is timely to revisit Arabidopsis research to examine how much has been achieved in the past, and how far the research of this model plant can contribute to crop improvement.

In general, two major traits are important to agriculture, namely, plant development and stress tolerance. Genes regulating plant development have been used in manipulating plant architecture, chemical composition, environmental responses, and senescence, while genes related to biotic (e.g., viral, bacterial, fungal, and animal attacks) and abiotic stresses (e.g., salt, heat, cold, and drought tolerance) are important for increasing stress tolerance. In this review, major discoveries that have contributed to our current understanding of these two processes are discussed, with emphasis on potential agricultural applications.

C.M. Liu
Center for Signal Transduction & Metabolomics (C-STM), Institute of Botany, Chinese Academy of Sciences, Nanxincun 20, Fragrant Hill, Beijing100093, China
e-mail: cmliu@ibcas.ac.cn

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2.2 Knowledge Gained in Arabidopsis Is Available for Crop Scientists

For plant geneticists, the advantage of using Arabidopsis as a research model, and sharing the knowledge generated is considerable. The fast propagation cycle (usually 30 to 60 days, depending on the photoperiod) allows genetic experiments to be performed in months instead of years, unlike the case for many annual and perennial crops. The large number of seeds produced provides sufficient progenies for statistical analyses. The small stature of the plant enables it to be grown in large numbers in small rooms with shelves. Arabidopsis has the smallest genome in flowering plants with a low gene redundancy, which facilitates mutant identification.

International collaborations have generated detailed genome information, extensive T-DNA knockout lines, and large-scale expression data. A comprehensive web-based community service center, The Arabidopsis Information Resource (TAIR: www.arabidopsis.org), has been established as a hub, maintaining an extensive dataset that is freely available for plant researchers. Data available from the TAIR database include complete genomic and transcriptomic sequences, along with gene structure, gene product information, metabolic pathways involved, seed stocks, T-DNA knockout lines, phenotypic and molecular markers, and publications. Other Arabidopsis-related resources, such as the Genvestigator (https://www.genevestigator. ethz.ch/), Electronic Fluorescent Pictograph (eFP: http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), and AtGenExpress Visualization Tool (AVT), provide extensive digital expression data based on microarray results (Zimmermann et al. 2004; Schmid et al. 2005; Winter et al. 2007). For almost every gene of interest, users may obtain T-DNA insertion knockout line(s), and microarray-based expression profiles from these databases.

2.3 Plant Architecture-Related Genes and Their Potential Uses in Crop Improvement

2.3.1 Genes Regulating the Function of Shoot Apical Meristem

The shoot apical meristem (SAM) integrates a diverse array of internal and external signals, and subsequently, adjusts its developmental program accordingly. It is a key area for cell division, organogenesis, and tissue differentiation, where new tissues and organs originate according to developmental phases. SAM comprises the central zone, where the stem cells and stem cell-organizing center are located, and the peripheral zone where different organ primordia are formed. Extensive intercellular communication is present in the shoot apex to regulate growth pattern and cell fate, particularly between the maintenance of stem cell population in the center, and the formation of predictable pattern of leaves at the periphery. Although these two functions occur in different regions of the meristem, their activities are

coordinated to maintain meristem integrity (Clark et al. 1993; Long et al. 1996; Mayer et al. 1998; Fletcher et al. 1999; Wu et al. 2005).

Signaling in the SAM was first characterized over 60 years ago by Sussex (1955). The results of his studies showed that existing leaf primordia determined future sites of organ formation in adjacent regions of the SAM. These findings laid the foundation for subsequent elucidation of the mechanisms controlling phyllotaxy. Liu et al. (1993) employed cultured zygotic embryos to demonstrate auxin polar transport defining the position of cotyledons. Using molecular markers in combination with surgical experiments, auxin in the SAM has been shown to be transported upward into the meristem through epidermal cells. The presence of auxin is directed from the existing leaf primordia to the regions where new leaf primordia are formed, creating its heterogeneous distribution in the meristem (Reinhardt et al. 2003). Auxin accumulation occurs only at certain distances from existing primordia, defining the position of future leaves. Recent studies on the characterization of *PHABULOSA-like* transcription factor genes have provided clues to the acquisition of cell identity in the newly formed leaves. These genes, which promote adaxial cell identity, are regulated by microRNAs (miRNAs; Tang et al. 2003; Golz 2006).

The CLV3-CLV1/CLV2 ligand-receptor complex, and the WUS transcription factor-based feedback regulation loop form a well-characterized signaling network in regulating meristem function (Fiers et al. 2007). *CLV3* of Arabidopsis encodes a small protein that can be processed into a functional 12-amino acid hydroxylated peptide from the conserved CLE domain (Fletcher et al. 1999; Fiers et al. 2005, 2006; Kondo et al. 2006; Ogawa et al. 2008). *CLV1* encodes a membrane-bound leucine-rich repeat (LRR)-receptor kinase, while *CLV2* encodes a LRR-receptor-like protein lacking a kinase domain (Clark et al. 1997; Jeong et al. 1999). The stem cells are accurately marked by *CLV3* expression, while the stem cell organizing centre (OC) is marked by expression of the homeodomain transcription factor *WUS*. The CLV3 peptide ligand interacts with a CLV1/CLV2 receptor complex to restrict the stem cell population in the SAM in a cell-autonomous manner, while WUS promotes the expansion of the stem cell population (Brand et al. 2000; Schoof et al. 2000). As such, all *clv* mutants (*clv1*, *clv2*, and *clv3*) possess enlarged SAMs, while the *wus* mutant or *CLV3* over-expressed plants are characterized by terminated SAM development (Laux et al. 1996; Hobe et al. 2003). Mutation of the *CLV3* ortholog of rice, *FON4*, leads to an increased number of rice floral organs, suggesting a functional conservation between dicotyledons and monocotyledons (Chu et al. 2006). From the application point of view, organ number, such as the number of leaves and carpels, is an important agricultural and horticultural trait. Identification of genes controlling organ number may provide a novel idea for crop design (see Chap.3 in this volume).

2.3.2 Lateral Organ Formation and Branching

Upon germination in most plant species, the shoot meristem passes through a juvenile phase, producing leaf primordia and stems sequentially (Poethig 2003;

Baurle and Dean 2006). During development, the SAM switches its program in response to environmental signals to make inflorescences and flowers. The arrangements of inflorescence and flowers in the reproductive phase are tightly regulated by inheritable patterns. Plant architecture is changed as a result of the formation of side shoots, e.g., tillers in rice, which often possess a growth pattern similar to that of the main shoot, arising from the axils of leaves. However, the timing and degree of growth of specific axillary meristems are controlled by various internal and external factors (Grbic and Bleeker 2000; Schmitz and Theres 2005; Beveridge 2006; Keller et al. 2006). Therefore, understanding the regulating networks of apical and lateral meristems may facilitate the redesign of plant architecture in crop species.

Several genes regulating the branching pattern have been identified in tomato. In the *lateral suppressor* (*ls*) mutant, the formation of lateral meristems is almost completely inhibited during vegetative development, while branching of inflorescences is reduced only slightly (Schumacher et al. 1999; Greb et al. 2003). Other tomato mutants of this group are *blind* (*bl*) and *torosa* (*to*), in which the formation of all lateral meristems is affected. The *Ls* gene has been shown to encode a protein of the GRAS family (Schumacher et al. 1999). Members of the GRAS family are plant-specific transcription factors that include the Arabidopsis genes *GIBBERRELIN INSENSITIVE* and *REPRESSOR OF ga1-3*, two negative regulators of GA signaling (Peng et al. 1997; Silverstone et al. 1998), and *SCARECROW* and *SHORTROOT*, which are the regulators of root development (Di Laurenzio et al. 1996; Helariutta et al. 2000). *Bl* and *To* are allelic. The *Bl* gene was isolated by positional cloning. It was found that the mutant phenotype was caused by a loss of function of an R2R3 class Myb gene. The identity of *Bl* was confirmed by RNA interference-induced *blind* phenotypes. This has resulted in a new class of transcription factors controlling lateral meristem initiation, and has revealed a previously uncharacterized function of R2R3 Myb genes (Muller et al. 2006).

Interestingly, the *MOC1* gene responsible for the *monoculm 1* (*moc1*) mutation phenotype in rice is the ortholog of tomato *Ls* and the Arabidopsis *LATERAL SUPPRESSOR* genes. The *moc1* plant produced a single culm without, or with a limited number of tillers, due to the loss of ability to initiate tiller buds (Li et al. 2003). All these genes have been shown to encode members of the plant-specific GRAS family proteins that function in various aspects of plant development, including signal transduction, and meristem maintenance and development (Richards et al. 2000; Bolle 2004). Like other members (RGA and SLR1) of the family, MOC1 is absent in typical nuclear localization sequences, but it is present in the nucleus, consistent with the hypothesis that MOC1 might function as a transcription factor (see Chaps. 4 and 5 in this volume).

2.3.3 Regulation of Stem Elongation

Auxins, GAs and brassinosteroids (BR) have been associated with cell elongation to determine plant height (Davies 2005). BR was discovered nearly 40 years ago

when Mitchell et al. (1970) reported that organic extracts of *Brassica napus* pollen promoted stem elongation and cell division in plants. However, convincing evidence of BR as an endogenous growth hormone was obtained only after the study of genetic analyses of several Arabidopsis dwarf mutants (Bishop and Yokota 2001; Kim et al. 2007). *Brassinosteroid insensitive1* (*bri1*) is a BR-insensitive dwarf mutant that carries recessive mutations in the *BRI1* gene. *BRI1* encodes a plasma membrane-associated LRR receptor kinase, indicating its role as a cell surface receptor for BL (Wang et al. 2001). Another putative receptor BAK1, which is an LRR receptor-like protein kinase, was later identified as a BRI1 interacting protein (Nam and Li 2002). Molecular and biochemical studies of these components have led to the establishment of a model for the BR signaling pathway, leading from BR perception at the cell surface to regulation of transcription in the nucleus. In the BR signaling pathway, BRs bind directly to the extracellular domain of BRI1 to activate its kinase activity, and promote heterodimerization with BAK1 and phosphorylation of BAK1. Other BR signaling components include glycogen synthase kinase-3-like kinase BIN2 as a negative regulator, and the nuclear proteins BZR1 and BZR2/BES1 as positive regulators. BIN2 negatively regulates BR signaling by phosphorylating and inhibiting BZR1 (He et al. 2002).

Apart from the semi-dwarf trait, another important gene, *GAI*, was identified in the Arabidopsis *GA insensitive* (*gai*) mutant (Peng et al. 1997). GAI and RGA, both belonging to the GRAS family, have overlapping functions as repressors of elongation growth (Peng et al. 1997; Silverstone et al. 1998). It later became clear that the semi-dwarf *RHT* gene in "Green Revolution" wheat was an ortholog of *GAI* (Peng et al. 1999). It is believed that the wild-type RHT proteins function as a negative regulator of GA signaling, and GA acts by repressing their functions, provided that the N-terminal domains are present. This is supported by results showing that ectopic expression of *GAI* in rice induced dwarfism, and loss-of-function mutations in *Rht*-like genes manifest in an overgrowth phenotype. This is another good example showing that model plants could assist gene identification in crops, such as wheat, with a complex genome structure.

In contrast to *RHT*, the semi-dwarf *sd-1* mutation leading to the rice "Green Revolution" is recessive, and normal height can be restored by exogenous application of GA, indicating that *sd-1* is defective in GA production. *sd-1* is one of the most important genes deployed in modern rice breeding. Its recessive character results in a shortened culm with improved lodging resistance and a greater harvest index, allowing for the increased use of nitrogen fertilizers. The *sd-1* gene was first identified in the Chinese variety Dee-geo-woo-gen (DGWG), and was crossed in the early 1960s with another high-yielding variety to develop the semi-dwarf cultivar IR8. IR8 produced record yields throughout Asia, and formed the basis for the development of new, high-yielding semi-dwarf plant types. Since the 1960s, *sd-1* has remained the predominant semi-dwarf gene present in rice cultivars (Hedden 2003). Three research groups have isolated the *SD-1* gene independently. *SD-1* was shown to encode GA 20-oxidase (GA20ox), which is a key enzyme involved in GA biosynthesis (Monna et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002). GA20ox was characterized 7 years earlier in Arabidopsis (Xu et al. 1995).

2.3.4 Regulation of Leaf Development

In plants, the number of leaves is tightly controlled to determining a longer or shorter vegetative phase (Baurle and Dean 2006; Corbesier and Coupland 2006). The switch from vegetative growth to flowering involves an integration of signaling pathways, resulting in the up-regulation of flowering genes. Activation of key integrators, *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF CONSTANS1*, results in a switch in shoot meristem identity, and the induction of flowering (Kardailsky et al. 1999; Kobayashi et al. 1999; Samach et al. 2000; Abe et al. 2005; Wigge et al. 2005). *FT* is expressed in leaves, but its mRNA appears to be a mobile signal that activates *APETALA1* (*AP1*) and *LEAFY* (*LFY*) on the flanks of the shoot meristem, thereby promoting floral meristem development (Mandel et al. 1992; Weigel et al. 1992; Hempel et al. 1997; Takada and Goto 2003; An et al. 2004; Huang et al. 2005). *TERMINAL FLOWER1* (*TFL1*), a temporal and spatial repressor of flowering genes, counteracts *FT* to influence each phase of growth (Shannon and Meeks-Wagner 1991; Alvarez et al. 1992; Schultz and Haughn 1993). Interestingly, these two proteins are also homologs (Bradley et al. 1997; Ohshima et al. 1997; Kardailsky et al. 1999; Kobayashi et al. 1999). Expression of *TFL1* is restricted to the inner cells of mature shoot meristems, and its mRNA level is very low during the vegetative phase, but is strongly up-regulated at the switch to flowering (Simon et al. 1996; Bradley et al. 1997; Ratcliffe et al. 1999). The results of a recent study showed that *TF1* encodes a mobile protein that is capable of moving in a few cell layers to surrounding cells in controlling Arabidopsis architecture (Conti and Bradley 2007). Components of RNA interference pathways are involved in defining the expression of genes that influence the identity of leaves (Hunter et al. 2006). Furthermore, a miRNA *miR160* has been targeted to the *auxin response transcription factor 17* (*ARF17*) to regulate the auxin response in Arabidopsis, defining many aspects of vegetative and reproductive growth (Mallory et al. 2005).

2.3.5 Regulation of Inflorescence Shape

In Arabidopsis, the shape of the inflorescence is regulated by many genetic factors, such as *ER*, *CLV1*, *CLV2*, and *CLV3*. *ER* encodes an LRR receptor kinase that regulates the elongation of the inflorescence stem (Torii et al. 1996), while *CLV1*, *CLV2*, and *CLV3* represent three genes controlling the numbers of floral organs in each flower, and the number of flowers in each inflorescence.

Cytokinin plays key roles in regulating plant architecture during growth and development. It has been shown to stimulate the formation and activity of shoot meristems, to retard leaf senescence, and to inhibit root growth and branching. Analysis of cytokinin-deficient plants suggested that the hormone has an essential function in the quantitative control of organ growth (Werner et al. 2001, 2003). A slight difference in organ size and number is sufficient to generate high-yielding crops. Ashikari et al. (2005) showed that, by modifying the structure of the panicle,

the quantitative trait locus *Gn1a* was responsible for increased grain productivity in rice. *Gn1a* encodes cytokinin oxidase/dehydrogenase (OsCKX2), an enzyme that degrades cytokinin. Reduced expression of *OsCKX2* resulted in cytokinin accumulation in inflorescence meristems, and an increase in the number of reproductive organs, leading to enhanced grain yield (Ashikari et al. 2005).

2.4 Understanding Abiotic Stresses to Improve Tolerance to Abiotic Stresses

2.4.1 Stress Responses

Abiotic stresses such as drought, high salinity, and cold are "unusual" environmental conditions that have an adverse effect on plant growth and productivity. However, during evolution, plants have developed a complex molecular machinery to cope with environmental stress. Studies in Arabidopsis have shown that many genes are highly responsive under stress conditions, and are expressed promptly in response to environmental triggers. Through detailed expression analyses, a list of 300 genes has been identified to be responsive to stresses (Fowler and Thomashow 2002; Seki et al. 2002). More than 50% of these genes are drought- and high salinity-inducible, suggesting a significant crosstalk between drought and high-salinity responses. In contrast, 10% of the drought-inducible genes could be induced by cold (Seki et al. 2002). These genes function not only in stress tolerance, but also in regulating downstream gene expression and signal transduction in response to stresses. Two groups of genes could be recognized under stress conditions. One shows rapid and transient expression, after drought, high-salinity, or cold stress, reaching a maximum after a few hours and then decreasing. These genes include the SOS2-like protein kinase PKS5, bHLH transcription factor, DREB1A, and DREB2A. Another group of genes expresses slowly, and increases gradually within 10 h after stress. Most of these genes encode functional proteins, such as LEA proteins. DREB1A and DREB2A are two transcription factors that are able to bind to the DRE-containing region (a 9-bp conserved sequence, TACCGACAT) of the promoters of several stress-responsive genes (Liu et al. 1998).

2.4.2 DREB Genes and Their Uses in Coping with Drought

DREB1s (also known as CBFs) represent a set of major transcription factors that regulate many cold-inducible genes. Transgenic Arabidopsis over-expressing *CBF1/DREB1B* under control of the *CaMV 35S* promoter showed a high tolerance to cold stress (Jaglo-Ottosen et al. 1998), while over-expressing *DREB1A* under the same promoter also increased the tolerance to drought, high-salinity, and freezing stresses (Kasuga et al. 1999). These transgenic plants also manifested a growth

retardation phenotype under normal growth conditions. However, over-expression of *DREB1A* under the stress-inducible *RD29A* promoter could minimize the negative effects on plant growth (Kasuga et al. 1999). The orthologs of *CBF*/*DREB1* have been reported in most crops examined to date, such as wheat, corn, rice, barley, canola, and soybean (Zhang et al. 2004a). Some of these orthologs have been successfully used to engineer abiotic stress tolerance in a number of crops, indicating conservation of the pathway in dicotyledons and monocotyledons. Over-expression of the Arabidopsis *CBF* genes has been shown to increase freezing tolerance in canola (Jaglo-Ottosen et al. 1998; Jaglo et al. 2001), whereas over-expression of the constitutive active form of *DREB2A* resulted in growth retardation in transgenic Arabidopsis. These transgenic plants showed significantly increased tolerance to drought stress, but only slight tolerance to freezing (Yamaguchi-Shinozaki and Shinozaki 2006). These genes are therefore potentially important to drought tolerance in crop species through genetic manipulation.

2.4.3 SOS Genes and Salt Tolerance

The salt overly sensitive (SOS) ion homeostasis and signaling pathway is another well-characterized abiotic stress response in Arabidopsis. The *SOS1*, *SOS2*, and *SOS3* loci were first identified through forward genetic screens for salt-hypersensitive growth. SOS1 is a plasma membrane Na^+/H^+ antiporter that is essential for Na^+ efflux from roots. SOS2 belongs to subgroup 3 of the sucrose non-fermenting-related kinases (SnRK3s). SOS3 is a myristoylated calcium-binding protein that likely responds to salt-induced Ca^{2+} oscillations in the cytosol. The SOS signaling pathway functions in regulating Na^+ homeostasis and salt tolerance in Arabidopsis. High Na^+ stress triggers a calcium signal that activates the SOS3-SOS2 protein kinase complex, which then stimulates the Na^+/H^+ exchange activity of SOS1 at the plasma membrane. SOS2 also activates Na^+/H^+ (AtNHX) exchangers on the vacuolar membrane (Zhang et al. 2004b). Increased expression of the Arabidopsis tonoplast membrane Na^+/ H^+ antiporter, *AtNHX1*, under a strong constitutive promoter was reported to result in salt-tolerant Arabidopsis (Apse et al. 1999), and in crops such as *Brassica napus* (Zhang et al. 2001) and tomato (*Lycopersicon esculentum*; Zhang and Blumwald 2001). Over-expression of *AgNHX1*, an *AtNHX1* ortholog from the halophytic plant *Atriplex gmelini* (Hamada et al. 2001), has led to improved salt tolerance in rice (Ohta et al. 2002).

2.5 Prospective Remarks

From the work discussed above, it is clear that the Arabidopsis research in the last 20 years has provided an extensive knowledge base for crop improvement. Some of the genes identified in Arabidopsis have already been exploited or evaluated in agro-biotechnological practice. Another systematically well-studied plant species is

rice, being both a model plant and a crop itself. As the number one staple food in the world, the advantage of using rice as a model is that the knowledge transfer from the laboratory to the field is much quicker.

Considering the limitations of Arabidopsis and rice, several new model plants have been proposed to dissect various biological processes that can not be studied in Arabidopsis. For instance, *Medicago truncatula* and *Lotus japonicus* have been used as models for legume crops to deal particularly with plant-*Rhizobium* interaction (Sato et al. 2007). *Brachypodium distachyon* has been selected as a model plant for cereal crops, especially for wheat, barley, and rye (Draper et al. 2001), while poplar (*Populus trichocarpa*) has been used as a model for woody plants (Jansson and Douglas 2007). It is believed that such community-based systematic studies of model plants, as demonstrated in Arabidopsis, will assist in gaining a thorough understanding of important biological processes, and their further exploitation in agricultural and horticultural practices.

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Part II
Vegetative Growth and Development

Chapter 3
Axillary Shoot Branching in Plants

M.W.F. Yaish, D.R. Guevara, A. El-Kereamy, and S.J. Rothstein

3.1 Introduction

Multiple shoot branching refers to the ability of a plant to produce an extra number of axillary shoots. This phenotype usually reflects healthy and yield-promising plants because increases in shoot branching can be translated to greater vegetative biomass, fruit and seed production. Historically, multiple shoot branches was a desirable trait in some crop plants, such as rice, in which multiple shoot branches (tillers) are associated with increased yield. In contrast, maize cultivars have been selected for a low number of axillary branches to improve the quality of the ears and kernels by concentrating plant resources.

High-yield production can be achieved by genetically altering the number of shoots per plant and/or by modifying other processes related to plant growth and development, as axillary branch formation is controlled by a complex interaction between genetically regulated developmental processes and the environment. Multiple shoot branching can also be achieved, to some extent, by augmenting the amount of fertilizers used in the field. However, increasing the fertilizer usage does not proportionally augment the yield because wild-type plants have a limited biochemical capacity to metabolize these artificially supplied inorganic nutrients. In fact, the application of high amounts of fertilizer to increase the number of shoot branches produced per plant would not only enhance input costs to farmers, but also lead to an accumulation of unused fertilizers in the soil which would ultimately pollute the groundwater. Therefore, the optimum situation is to use reasonable amounts of supplied nutrition and to genetically alter the number of shoot branches

M.W.F. Yaish
Department of Biology, College of Science, Sultan Qaboos University, P.O. Box: 50, 123 Muscat, Oman
e-mail: myaish@squ.edu.om

D.R. Guevara, A. El-Kereamy, and S.J. Rothstein
Department of Molecular and Cellular Biology, University of Guelph, Guelph N1G 2W1, Ontario, Canada

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to obtain the desired plant architecture to maximize yield in crop plants. This requires an understanding of the mechanisms controlling plant architecture.

Significant progress has been made towards gaining a better understanding of the mechanisms responsible for axillary meristem initiation and development due, in part, to the availability of modern reverse genetics and genetic mapping technologies. Reverse genetic approaches, which are based on determining the phenotypic effect of losing a functional gene, have facilitated the identification of genes involved in multiple branching phenotypes. Genes identified so far using these technologies have been shown to display different degrees of regulatory relationships with known branching mechanisms, and to encode products involved in hormonal mobilization, gene transcription, protein ubiquitation or degradation networks. Discoveries achieved in the area of shoot branching physiology were previously thoroughly discussed in several excellent reviews which cover topics such as the physiology of secondary bud initiation (Prusinkiewicz 2004; McSteen and Leyser 2005; Dun et al. 2006), the role of hormones in shoot branching (McSteen and Leyser 2005; Doust 2007; De Smet and Jurgens 2007; Ongaro and Leyser 2008) and the genes involved in shoot branching (Wang and Li 2006; Doust 2007). In this chapter, we discuss the progress achieved so far in understanding shoot branching mechanisms in plants and the metabolic pathways controlling this process.

3.2 Axillary Shoot Development

The initiation of axillary branching and development is a complicated process and was found to be controlled by a range of genes. The identification of genes controlling this process is crucial since they would provide key targets which can be manipulated to improve plant architecture in order to increase crop yield. Most of the genes involved in the initiation of axillary branching were discovered by studying the phenotypic effect of a mutation within a given gene. Therefore, in some cases there is an indirect relationship between these genes and the actual axillary branching mechanism. Nevertheless, the role which these genes play in controlling this process and the other factors which these genes influence have been thoroughly studied. For example, the genes discovered so far were found to control branching through alteration of different transcriptional or hormonal pathways. Also, different types of transcription factor genes were characterized to be keys in regulating these pathways by controlling the expression of gene networks involved in axillary branching. This indicates that the coordinated expression of an array of genes may be necessary in order to achieve multiple branching phenotypes. In addition, other identified proteins implicated in axillary branching are involved mainly in hormonal regulation pathways, suggesting that hormones are vital for the regulation of axillary branching. This chapter discusses the recently identified genes which show significant effects on axillary branching. It is possible that, at a certain stage of bud initiation and development, both wild-type and multiple axillary mutants share a common molecular pathway. After this point, other factors are responsible for leading to the observed differentiation in axillary

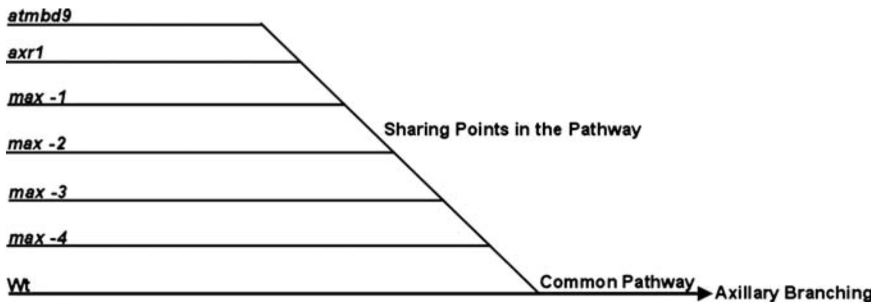

Fig. 3.1 Genetic modifications lead to multiple axillary branching in Arabidopsis. Schematic representation of the proposed axillary branching pathways involved. The different genotypes share a common pathway of axillary bud initiation and outgrowth at some point

branch numbers giving rise to the multiple branching phenotype in the mutants thus far studied (Fig. 3.1). The known information on these genes is discussed, as well as the possible pathways in which they are involved.

3.2.1 Bud Initiation

Based on available information, plant species share some similarities in their control of shoot initiation. In dicotyledons, plant growth stops once a genetically predetermined structure has completely formed (determinately), or can continue to develop throughout the life of a plant (indeterminately). The meristem produces phytomers, which are shoot units consisting of a leaf, axillary bud and a stem segment. Arabidopsis mutant plants of unfunctional Terminal Flower1 (TFL1) transcription factor (Shannon and Meeks-Wagner 1991) have a determinant meristem. TFL1 controls the growth of the phytomer by delaying the expression of floral-related genes, such as *LEAFY* (*LFY*) and *APETALA1* (*AP1*), and hence gives more time for the vegetative axillary buds to initiate and grow (Ratcliffe et al. 1999). Determination of the mechanism by which an axillary bud initiates and develops is important to genetically design plants with a desired branching habit. In fact, there are mainly two different hypotheses describing the process of shoot apical meristem initiation in dicotyledons (McSteen and Leyser 2005). The first is that the new apical meristem starts to form and grow at the leaf axils (Snow and Snow 1942). The second is that the apical meristem results from the growth of active clusters of meristem cells which were originally present in the apical meristem at the time of leaf initiation (Garrison 1955; Sussex 1955). A new hypothesis on axillary bud initiation, which merges the first and the second hypotheses, has been developed based on the molecular functional characterization of a gene which encodes for the transcription factor LATERAL SUPRESSOR (LS) in tomato (Schumacher et al. 1999) and its ortholog of Arabidopsis (LAS; Greb et al. 2003). It is postulated that the LS/LAS prevents the complete differentiation of the leaf axil and thereby maintains its meristematic potential.

In monocotyledons, vegetative branches are called tillers, and usually arise from the basal node of the first formed phytomer. Beside tillers, grasses produce secondary or axillary branches which hold the ears, as in maize. Loss of function of the *MONOCULM1* (*MOC1*) gene (Li et al. 2003), an ortholog of *LS*/*LAS*, resulted in absence of tillers in rice, providing strong evidence of a common shoot branching mechanism in monocotyledons and dicotyledons. Overexpression of the *MOC1* gene increases the number of tillers, implying that this gene can promote apical meristem outgrowth and initiation. A similar defect was noted in the *uniclum2* (*clu2*) barley mutant, in which mutant plants lacked tillers (Babb and Muehlbauer 2003). However, in this case, the defect does not affect apical meristem initiation but cannot guarantee their meristematic activity and development. In contrast, *TEOSINTE BRANCHED 1* (*TB1*) genes in maize (Doebley et al. 1997; Wang et al. 1999; Hubbard et al. 2002) and its ortholog in rice (*OsTB1*; Takeda et al. 2003) suppress tiller and branch growth. Mutations leading to loss of function in these genes result in multiple axillary branch development in maize and a greater tiller number in rice. Despite these valuable findings on genes which control apical meristem initiation in monocotyledons, other genes need to be identified and characterized, because important information is required to clarify some of developmental processes during apical meristem initiation and development. The involvement of other key genes is not unexpected, given the occurrence of significant QTLs other than the *TB1* loci in a genome of some species, such as foxtail millet (Doust et al. 2004).

3.2.2 Genes Control Axillary Shoot Branching

Tremendous progress has been achieved recently towards the identification of new genes which influence axillary shoot branching. These genes have been identified mainly in Arabidopsis or rice plants. However, because axillary branching pathways are common amongst plants, this allows isolation of new genes from different species. Several transcription factors have been found to control axillary branches (Table 3.1). Amongst these transcription factors are the tomato *Blind* (*BL*) gene which encodes for a myb transcription factors (Schmitz et al. 2002), the Arabidopsis *AtMBD9* which encodes for a methyl-CpG binding domain (Peng et al. 2006; Yaish et al. 2009), and the rice *OsNAC2* which codes for a NAC (NAM, ATAF1, 2, CUC2) transcription factor family protein (Mao et al. 2007). These transcription factors have the opposite effect on enhancing axillary shoot formation in plants. While a functional BL protein is required for normal lateral meristems in tomato, and the overexpression of the *OsNAC2* in rice plant increases the number of tillers, mutations within *AtMBD9* also lead to an increase in the axillary branches in Arabidopsis. This indicates the indirect functional control of these transcription factors. Therefore, this fact implies the need to identify the downstream gene products which are controlled by these transcription factors.

Table 3.1 Genes involved in shoot branching

Gene name	Plant species	Class	Function	Reference
AUXIN-RESISTANCE (*AXR1*)	Arabidopsis	Ubiquitin-activating enzyme e1	Controls auxin response	Leyser et al. (1993)
BLIND (*BL*)	Tomato	Transcription factor	Regulation of apical meristem initiation	Schmitz et al. (2002)
BRANCHED1 (*RRC1*)	Arabidopsis	Transcription factor	Prevents axillary bud formation	Aguilar-Martínez et al. (2007)
BUSHY AND DWARF1 (*BUD1*)	Arabidopsis	Kinase	Controls auxin polar transportation	Dai et al. (2006)
DECREASED APICAL DOMINANCE1 (*DAD1*)	Petunia	Dioxygenase	Controls branching	Snowden et al. (2005)
LATERAL SUPPRESSOR (*LAS*)	Arabidopsis	Transcription factor	Controls axillary meristem formation	Greb et al. (2003)
LATERAL SUPPRESSOR (*LS*)	Tomato	Transcription factor	Controls initiation of axillary meristems initiation	Schumacher et al. (1999)
METHYL-CPG BINDING9 (*AtMBD9*)	Arabidopsis	Transcription factor	Controls axillary branching	Peng et al. (2006), Yaish et al. (2009)
MONOCULM1 (*MOC1*)	Rice	Transcription factor	Controls tiller initiation and outgrowth	Li et al. (2003)
MORE AXILLARY GROWTH1 (*MAX1*)	Arabidopsis	Cytochrome P450	Repressor of vegetative bud outgrowth	Stirnberg et al. (2002), Greb et al. (2003), Booker et al. (2005)
MORE AXILLARY GROWTH2 (*MAX2*)	Arabidopsis	F-box LRR	Involvedx in max. signalling pathway	Stirnberg et al. (2002), Greb et al. (2003)
MORE AXILLARY GROWTH3 (*MAX3*)	Arabidopsis	Dioxygenase	Catalyzes the biosynthesis of carotenoid-derived regulators of axillary bud outgrowth inhibitors	Booker et al. (2004)
MORE AXILLARY GROWTH4 (*MAX4*)	Arabidopsis	Dioxygenase	Involved in the biosynthesis of carotenoid-derived axillary bud inhibitors	Sorefan et al. (2003)
NAC (*NAM*, *ATAF1*, *2*, *CUC2*) (*OsNAC2*)	Rice	Transcription factor	Controls tillering	Mao et al. (2007)

(*continued*)

Table 3.1 (continued)

Gene name	Plant species	Class	Function	Reference
RAMOSUS (*RMS*)	Pea	Dioxygenase	Regulates shoot branching	Sorefan et al. (2003)
REVALUTA (*REV*)	Arabidopsis	Transcription factor	Regulation of apical meristem initiation	Otsuga et al. (2001)
SUPPERSHOOT (*SPS*)	Arabidopsis	Cytochrome P450	Involved in axillary bud initiation and growth	Tantikanjana et al. (2001)
TEOSINTE BRANCHED1 (*OsTB1*)	Rice	Transcription factor	Negatively regulates lateral branching	Takeda et al. (2003)
TEOSINTE BRANCHED1 (*TB1*)	Maize	Transcription factor	Controls lateral bud outgrowth	Doebley et al. (1997)

In addition to transcription factors, hormone-related proteins have been shown to have a direct effect on axillary branches. Classically, auxin was known to control axillary branches through the apical dominance phenomena. Therefore, loss of the apical meristem usually leads to increases in the number of axillary branches. Mutation within proteins involved in auxin polar transportation leads to a dwarf and bushy phenotype in the *bud1* mutant (Dai et al. 2006). Likewise, loss of function of the Arabidopsis *AUXIN-RESISTANCE* (*AXR1*) gene reduces the response of Arabidopsis to auxins and increases the axillary branches in Arabidopsis (Leyser et al. 1993).

More recently, novel hormone-like molecules controlled by a group of genes know as *MAXIMUM AXILLARY GROWTH* (*MAX1-4*) were identified and found to be involved in the synthesis and transportation of a non-classical growth regulator, carotenoid-derived signalling molecules (Stirnberg et al. 2002). The *MAX* gene family has homologs in pea *RAMOSUS* (*RMS*; Sorefan et al. 2003) and in petunia *DECREASED APICAL DOMINANCE1* (*DAD1*; Snowden et al. 2005).

3.3 Hormones Involved in Axillary Bud Formation

Shoot branching is determined by the outgrowth of axillary buds, which is regulated by a wide range of endogenous and environmental factors. The most important endogenous factors are the plant hormones. So far, three hormones are known to be involved in axillary bud outgrowth and, consequently, shoot branching. These hormones include auxin and cytokinin, as well as new, chemically unidentified metabolite-like hormones. The following section highlights the different proposed models for the hormonal network-regulated shoot branching.

3.3.1 Auxin, Cytokinin and Novel Hormone

The physiological role of auxin and cytokinin in shoot branching has been studied extensively. Auxin is the first plant hormone shown to be involved in shoot branching, and it has been established that it controls the shoot tip apical dominance and, consequently, inhibits axillary bud outgrowth. Additionally, the replacement of the shoot apex with exogenous auxin maintains the inhibition of axillary buds (Cline 1996). Cytokinins show the opposite physiological role to auxin, since they act directly to promote axillary bud outgrowth. Studies have demonstrated that either exogenous cytokinin application or overexpression of genes encoding enzymes involved in cytokinin biosynthesis often induce bud outgrowth (King and Van Staden 1988; Medford et al. 1989; Miguel et al. 1998). In addition, some of the mutants with a greater level of cytokinin show more shoot branching (Dun et al. 2006).

Another carotenoid-like plant hormone with as yet unknown chemical structure was proposed to be involved in regulating bud outgrowth, by the analysis of the

branching mutants in Arabidopsis, pea and petunia. It was shown that the loss of function of the *MAX1*, *MAX2*, *MAX3*, *MAX4*, *MAX5* in Arabidopsis, *RMS1*, *RMS2*, *RMS3*, *RMS4*, *RMS5* and *RMS6* in pea, or *DAD1*, *DAD2*, *DAD3* in petunia resulted in increasing the shoot branching compared to the wild types (Rameau et al. 2002; Stirnberg et al. 2002; Sorefan et al. 2003; Bennett et al. 2006). Most of the *MAX*, *RMS* and *DAD* genes have been cloned and appeared to be orthologous (reviewed in Ongaro and Leyser 2008).

3.3.2 Axillary Bud Outgrowth Hypotheses

Three hypotheses were proposed for the role of the plant hormones auxin and cytokinin in shoot branching (Dun et al. 2006). These are the classical hypothesis, the auxin transport hypothesis and the bud transition hypothesis. The classical hypothesis proposed that auxin regulates shoot branching by influencing the level of other signals required for bud outgrowth inhibition (Dun et al. 2006). These signals are referred to as second messengers for auxin action (McSteen and Leyser 2005). Evidence for the role of second messengers was obtained from various studies which found a link between the cytokinin biosynthetic pathway and bud outgrowth. For example, decapitation in legumes resulted in a concomitant increase in the endogenous cytokinin concentrations in axillary buds, possibly mediated by an increase in the expression of the cytokinin biosynthesis genes (isopentenyl transferase IPT1 and IPT2) in the stem. This increase is partially removed by auxin application (Tanaka et al. 2006) and, consequently, reduces the cytokinin supply to the bud (McSteen and Leyser 2005). It was suggested that novel hormone, in addition to cytokinin, might serve as second messenger for auxin action (McSteen and Leyser 2005).

The second hypothesis is based on the auxin transport stream. Auxin is synthesized in the apical meristems and transported to the basal plant organs thorough the polar auxin transport stream (Ljung et al. 2001). It was suggested that this stream is saturated with auxin and thus prevents the flow of the auxin from the axillary buds in the plants where the axillary bud outgrowth is inhibited (Li and Bangerth 1999; Leyser 2005). Several proteins are involved in active auxin transport. In *Arabidopsis*, the main shoot PIN1 (Auxin efflux carrier) appears to be particularly important for polar auxin transport (Okada et al. 1991). Supporting the transport hypothesis, Bennett et al. (2006) reported a higher level of labelled auxin and PIN1 expression in the stem of the *max4* mutant. Similar results were observed in the *rms* pea mutant (Beveridge et al. 2000). Enhancing auxin movement in the branching mutants may be an indicator for the auxin flow enhancement in these plants, which is responsible for the increased shoot branching. However, the shoot endogenous auxin content may not have a direct correlation with shoot branching. For example, grafting *rms2* scion on the wild-type rootstock inhibits bud outgrowth without reducing a high auxin concentration in the *rms2* internodes, which might be due to the feedback regulation which allows the signals from the wild-type region to inhibit bud outgrowth (Foo et al. 2005; Morris et al. 2005; Beveridge 2006).

The third hypothesis for auxin in shoot branching is the bud transition hypothesis. Based on this hypothesis, bud development can be classified in three stages: dormancy, transition and sustained growth (reviewed in Dun et al. 2006). It seems that bud location on the stem influences its outgrowth potential and its response to cytokinin or to decapitation. For example, cytokinin application is effective in inducing the outgrowth of the axillary buds at pea node 2. However, this treatment does not promote the growth of the axillary buds at node 3 or node 4 (King and Van Staden 1988). It was proposed that bud growth is determined by the bud stage, and the auxin can act to inhibit the bud outgrowth only in the transition stage. This hypothesis is supported by the findings of Morris et al. (2005), who reported the occurrence of a rapid signal which led to the dormant bud entering into the transition stage after decapitation. This includes the initial but not the sustained bud growth. Thus, the current understanding can be integrated with the classical hypothesis, which proposes that auxin may inhibit the growth of the bud in the transition stage by affecting the cytokinin response.

3.3.3 Abscisic Acid and Branching

It is well known that "cross talk" exists in the hormonal networks which are involved during different developmental stages throughout the plant's life cycle. Therefore, in addition to the well-known role of auxin and cytokinin, we cannot exclude the possibility of the participation of other hormones such as abscisic acid (ABA), also a carotenoid derivative, in controlling axillary bud outgrowth and, consequently, shoot branching. Several studies have been carried out to elucidate the role of the plant hormone ABA in shoot branching and its interaction with auxin. To date, the precise role of this hormone in the branching network is not clear. ABA was also implicated as a secondary messenger which modulates auxin-induced repression of axillary bud growth. However, evidence to support this is lacking (Chatfield et al. 2000). The possible role of ABA in controlling axillary bud outgrowth is supported by the fact that ABA is a "dormancy hormone", and the exogenous ABA application inhibits the growth of active buds. Decapitation is also accompanied by a reduction of the lateral bud ABA content (Geuns et al. 2001). For example, the increase of endogenous indole-3-acetic acid (IAA) at the terminal buds and internodes of soybeans, when exposed to shaded light of a low red:far-red ratio, induced an increased synthesis of ABA in the axillary buds (Begonia and Aldrich 1990). Also, the ABA-insensitive *AB13* mutant inhibited vegetative growth and was expressed abundantly in dormant axillary buds (Rohde et al. 1999 http://aob.oxfordjournals.org/cgi/content/full/98/4/ - B28). Work on the ABA-insensitive *Arabidopsis* mutants, *abi1-1* and *abi2-1*, demonstrated that auxin inhibition of axillary bud outgrowth is ABA-independent and excludes the involvement of ABA in apical dominance (Chatfield et al. 2000). Furthermore, compared to wild type, the leaves of the pea *rms2* mutant are similar in ABA content and responses to ABA on stomatal conductance (Dodd et al. 2008). Interestingly, recent work using

decapitated shoots of *Ipomoea nil* (Japanese morning glory) and *Solanum lycopersicum* (Better Boy tomato) revealed that, unlike auxin, apically applied ABA did not restore apical dominance, but ABA was able to repress lateral bud outgrowth when applied basally (Cline and Oh 2006). These findings imply a possible interaction between ABA, auxin and the unidentified carotenoid-derived hormone, whereby ABA is able to restore apical dominance via acropetal transport up the shoot (Cline and Oh 2006). The finding opens up new avenues of investigation on the role of ABA in apical dominance. Thus, despite the evidence for the involvement of ABA in the inhibition of the axillary bud outgrowth, details about its role and its interaction with auxin and cytokinin still need further clarification.

3.4 Regulatory Pathways Involved in Shoot Branching

Shoot system architecture is regulated by the establishment of axillary meristems and the outgrowth of axillary buds. While auxin is the primary effector of shoot branching, auxin does not enter the lateral buds to inhibit bud growth. Instead, other secondary messengers are involved in the repression of bud outgrowth, and their actions are mediated by auxin. This section describes the diverse set of molecules which interact with auxin to control the shoot system architecture.

3.4.1 Carotenoid-Derived Signalling Molecules

Carotenoids are a class of isoprenoid-derived compounds which are produced in the plastids. Carotenoids can absorb light energy and dissipate excess energy, and are precursors for hormone biosynthesis. A novel carotenoid-derived compound with unknown chemical structure has been shown recently to be required for the inhibition of axillary bud growth. This was demonstrated through the analysis of the *DAD1*, *MAX4* and *RMS1* mutants in petunia, *Arabidopsis* and pea respectively, which displayed an increase in lateral branching (Sorefan et al. 2003; Snowden et al. 2005; Bennett et al. 2006). The *DAD1*, *MAX4* and *RMS1* mutants result from lesions in the gene which encodes a carotenoid-cleavage dioxygenase (CCD). Therefore, the increase in branching in these mutants is due to the inability to synthesize a carotenoid-derived signalling molecule capable of inhibiting axillary meristem development (Schwartz et al. 2004; Bennett et al. 2006).

The carotenoid-derived signalling molecule is synthesized via the *MAX* (*More axillary branching*) pathway in *Arabidopsis*. There are four genes (*MAX1* to *MAX4*) in this pathway, and the synthesis of the acropetally mobile molecule depends on the actions of MAX1, AtCCD7 (MAX3) and AtCCD8 (MAX 4). Analysis of the recombinant proteins showed that AtCCD7 catalyzes a 9–10 cleavage of β-carotene to produce the 10′-apo-β-carotenal and β-ionone, while AtCCD9 catalyzes a 13–14 cleavage of the 10′-apo-β-carotenal to produce 13′-apo-β-carotenone (Schwartz

et al. 2004). *MAX1* encodes a cytochrome P_{450} and acts downstream of MAX3 and MAX4. *MAX2* encodes an F-box LRR family protein and is responsible for perceiving the signal. It has been proposed that the MAX-dependent pathway branching signal interacts with auxin and cytokinin hormone networks (Wang and Li 2008).

In addition to the MAX-dependent pathway branching signal, another carotenoid-derived signalling molecule has been identified based on work done on the *bypass1* (*bps1*) *Arabidopsis* mutant. The *bps1* mutant displayed loss of shoot apical meristem activity as a result of a constitutively produced graft-transmissable signal capable of arresting shoot growth (Van Norman and Sieburth 2007). The synthesis of this signal requires β-carotene but not the activity of CCDs and, therefore, does not require AtCCD7 or AtCCD8.

Taken together, it is clear that the carotenoid pathway is important for the synthesis of mobile signals which regulate shoot development. The next goal is to determine the chemical structure of these novel signalling molecules in order to examine the mechanism involved in regulating shoot branching. Since these carotenoid-derived signalling molecules also move acropetally from the roots to the shoots, and modulate auxin-mediated repression of bud outgrowth, it will also be important to determine whether these novel carotenoid-derived signals interact with ABA to modulate auxin-mediated repression of bud growth.

3.4.2 Polyamines

Polyamines are aliphatic nitrogen compounds implicated in playing important roles in plant growth and development. The involvement of polyamines in apical dominance was demonstrated using *isopentyl transferase* (*ipt*)-transformed tobacco. It was observed that the defoliation of upper nodes of *ipt*-transformed tobacco plants led to an enhanced concentration of cytokinins in the axillary buds. This resulted in the release of the axillary buds from dormancy, and a concomitant change in polyamine composition occurred, whereby putrescine and spermidine levels decreased and spermine levels increased in the axillary buds (Geuns et al. 2001). It has been proposed that polyamines may play an important role in the subsequent growth and development of axillary buds into shoots after their release from dormancy (Geuns et al. 2001). Similar patterns have been observed in other plants. For example, the *Arabidopsis* bushy and dwarf mutant, *bud2*, shows severe alterations in apical dominance. The *bud2* mutant results from the complete deletion of the gene which encodes an *S*-adenosylmethionine decarboxylase (SAMDC; Ge et al. 2006). This SAMDC is required for the synthesis of the polyamines spermidine and spermine from putrescine. Consequently, the *bud2* mutant had higher levels of putrescine and lower levels of both spermidine and spermine, and this alteration in polyamine homeostasis led to the termination of dormancy of axillary buds. However, the response of *bud2* to auxin and cytokinin remains to be determined. Further work on this mutant may provide insights into the precise role polyamines play in shoot branching (Ge et al. 2006).

Recent work by Falasca et al. (2008) showed that spermidine, putrescine and α-1,4-linked oligogalacturonides (OGs) enhanced the formation of cytokinin-induced adventitious vegetative shoots in tobacco leaf explants. The effect of putrescine was less pronounced than that of spermidine. However, unlike spermidine, the effect of OG on the enhancement of adventitious vegetative shoot formation was calcium-independent, and the stimulatory effect of spermidine was enhanced in the presence of auxin (Falasca et al. 2008). Moreover, exogenous application of calcium and auxin to tobacco leaf explants led to an enhancement in the expression of genes encoding enzymes involved in polyamine biosynthesis, whereas exogenous OG repressed their expression. This implies that while polyamines affect cytokinin-induced vegetative shoot regeneration, calcium and auxin may modulate their effects during shoot growth (Falasca et al. 2008). Therefore, future work should take into account the interplay between auxin, cytokinin, OGs and calcium in mutants defective in polyamine biosynthesis to determine their importance and the mechanism controlling plant architecture.

3.4.3 Inositol Phosphates

Inositol phosphates (IPs) are a group of phosphorylated C6-cyclitols, and are important secondary messengers in eukaryotic cells. For example, inositol 1,4,5-triphosphate (IP_3) and inositol 1,3,4,5-tetrakis-phosphate (IP_4) are secondary messengers which regulate cytosolic calcium concentration in animal cells (Berridge 1993). In *Arabidopsis*, the inositol polyphosphate 6-/3-kinase genes (*AtIpk2a* and *AtIpk2β*) encode enzymes capable of converting IP_3 to inositol 1,4,5,6-tetrakis-phosphate, a precursor for phytate synthesis (Stevenson-Paulik et al. 2002). Recently, *AtIpk2β* has been shown to play a role in axillary shoot branching by controlling auxin signalling (Zhang et al. 2007). *Arabidopsis* plants with the overexpressed *AtIpk2β* gene possessed more axillary shoot branches, and had greater bud outgrowth rates compared to wild type (Zhang et al. 2007). Moreover, *Arabidopsis* plants with the overexpressed *AtIpk2β* gene had repressed levels of the *MAX4* transcript. Interestingly, *AtIpk2β* was induced by exogenous auxin, and *AtIpk2β* overexpression lines displayed altered auxin responses, as well as deviations in auxin distribution and accumulation. Therefore, these findings strongly imply that *AtIpk2β* regulates axillary shoot branching in *Arabidopsis* by interacting with the auxin-signalling pathway and the *MAX*-dependent pathway branching signal.

The role that *AtIpk2β* plays in axillary branching via auxin signalling is intriguing. The recent determination of the crystal structure of the auxin receptor TIR1 supports a further role for phytate, the end product derived from IP_3 via actions of AtIpk2β. The auxin-binding pocket of TIR1 is stabilized by phytate (Kepinski 2007), and this finding provides insights into the mechanism controlling auxin-mediated signalling via the actions of *AtIpk2β*.

In conclusion, recent work has demonstrated that secondary messengers are crucial for auxin-mediated repression of bud outgrowth which shapes plant architecture. Future work should focus on the interplay between secondary messengers and the hormone networks which modulate their activity to unravel the mechanism controlling shoot branching.

3.5 Future Perspectives

Multiple axillary branching should be considered for increasing crop biomass formation and yield. Engineering plants with maximum axillary shoot number is not a simple task because several mechanisms involved are still unclear. Also, the exact function of auxin and cytokine receptors, as well as the nature of the *MAX* gene products and their role in inducing axillary buds need further investigation. The information released from high-throughput microarray and metabolic pathway data, along with additional genetic and physiological studies, may better clarify the axillary shoot branching processes.

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Chapter 4
Bud Dormancy and Growth

D. Horvath

4.1 Introduction

Buds are the primary shoot-producing meristematic organs for dicotyledonous plants, and thus play the key role in growth, reproduction, and architecture. Buds often form at the axils of leaves, but can also form adventitiously on the stem, leaves, crown (or underground portion of the hypocotyl) or roots. Buds can be committed to either vegetative or floral development upon formation, or can shift after formation from vegetative to floral development depending on environmental and endogenous signals. Given their powerful role in plant growth and development, it is not surprising that bud formation and growth is a tightly controlled and complexly orchestrated phenomenon.

For buds, growth is the default program, and thus, nearly all of the mechanisms that regulate bud growth primarily act to prevent growth by initiating and maintaining bud dormancy. Lang et al. (1987) described several non-overlapping processes that control bud dormancy. They identified the dormancy states as paradormancy, endodormancy, and ecodormancy. In temperate regions of the world, these three dormancy states are commonly associated with seasonal transitions. In paradormancy, signals produced in other parts of the growing plant primarily inhibit bud growth. Paradormancy is also known as apical dominance, or correlative inhibition. The level of axillary and adventitious bud paradormancy during the growing season is often relative, and controlled release from paradormancy often dictates the general bushiness and architecture of the plant. During endodormancy, sometimes referred to as innate dormancy, buds will not grow even if they are not correlatively inhibited and environmental conditions are conducive to growth. Endodormancy is best studied in perennial plants from temperate environment,

D. Horvath
United States Department of Agriculture-Agricultural Research Station, Biosciences Research Laboratory, 5674 State University Station, Fargo, ND 58105-5674, USA
e-mail: horvathd@fargo.ars.usda.gov

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where endodormancy often manifests itself in the fall and is required to prevent bud growth during brief periods of warm weather that might occur in late fall or early winter. Ecodormancy occurs when environmental conditions, such as extreme cold or drought, prevent bud growth. Although others have proposed different systems and definitions of dormancy over the years, molecular studies show that distinct signaling pathways appear to regulate the different dormancy states (Lang et al. 1987).

4.2 Regulation of Paradormancy

Darwin and Darwin (1880) first identified the likelihood that there was a signal produced in growing shoot apices of a plant that could inhibit the growth of distal buds. Later, this signal was shown to be the plant hormone auxin (see Cline 1994). Many experiments demonstrated that blocking auxin production or transport from the shoot apices released distal buds from paradormancy (Cline 1994). However, controversy over the role of auxin in apical dominance continues. Apically produced auxin does not accumulate in growth-repressed buds. Consequently, a hypothesis that auxin was acting through a secondary messenger was developed to explain this discrepancy.

4.2.1 Hormonal Control of Paradormancy

Cytokinins have long been implicated in the regulation of bud dormancy (Cline 1994). Stafstrom and Sussex (1988) demonstrated that cytokinin treatment induced protein accumulation patterns similar to those observed following paradormancy loss in pea buds. Shimizu-Sato and Mori (2001) demonstrated that loss of auxin transport increased cytokinin production in the nodes proximal to axillary buds, and that cytokinin was transported into the buds (Fig. 4.1). Additionally, there is evidence that auxin directly regulates the production of cytokinin through the action of AXR1/TIR1/AFB and other auxin signaling mechanisms that directly impact the expression of cytokinin biosynthesis genes (reviewed in Ongaro and Leyser 2008). Cytokinin enhances bud growth, and directly enhances cell division through activation of key cell cycle regulatory genes such as CYCLIN D (Riou-Khamlichi et al. 1999). Shimizu-Sato and Mori (2001) also noted that apically transported auxin enhanced the expression of genes involved in abscisic acid (ABA) accumulation in nodes proximal to the bud. ABA may inhibit cell division through induction of ICK1/KRP1 (Wang et al. 1998), a protein that inhibits the G1 to S phase transition of the cell cycle. The mechanism by which auxin activates these processes is not known. However, mutations in genes regulating these phenomena have been identified, and are beginning to shed light on the signaling pathways.

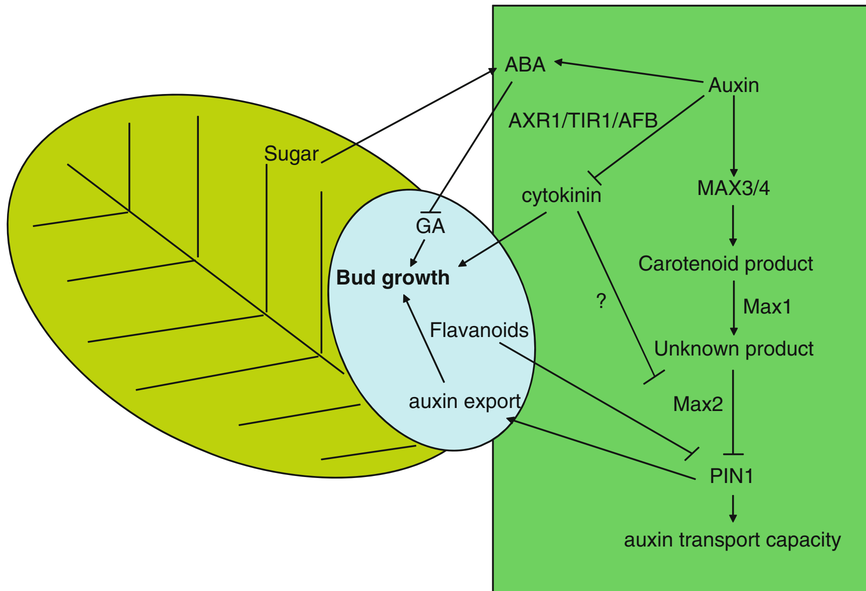

Fig. 4.1 Schematic representation of an internode showing the stem (*dark*), leaf (*light*), and bud (*intermediate*), along with various signals and their interactive responses that influence bud growth. ABA, abscisic acid; AXR1, AUXIN RESPONSIVE1; TIR1, TRANSPORT INHIBITOR RESPONSE1; AFB, AUXIN RECEPTOR F-BOX PROTEIN; GA, gibberellic acid; MAX, MORE AXILLARY BRANCHING; PIN1, PIN-FORMED1

4.2.2 The RMS/MAX/DAD System Regulates Bud Dormancy

The laboratories of Beveridge, Leyser, and Snowden have identified the *RMS/MAX/DAD* pathways in pea (*Pisum sativum*), arabidopsis (*Arabidopsis thaliana* Heyn), and petunia (*Petunia* x *hybrida*), respectively, as a generally conserved signaling pathway regulating auxin-dependant bud outgrowth (reviewed in Ongaro and Leyser 2008). Briefly, *RAMOUS1* (*RMS1*), *MORE AXILLARY GROWTH4* (*MAX4*), and *DECREASED APICAL DOMINANCE1* (*DAD1*) are genes that, when mutated, result in increased branching that could not be rescued by addition of exogenous auxin. Although all these genes likely perform a similar function, they are not all regulated in identical manner in their respective species.

MAX4 appears to be constitutively expressed in arabidopsis, whereas *RMS1* and *DAD1* appear to have altered expression following the loss of polar auxin transport in pea, or when present in combination with other related mutations in pea and petunia. *RMS1/MAX4/DAD1* encodes a CAROTENOID CLEAVAGE DIOXYGENASE (CCD) protein. The CCD protein is required to produce a strigolactone that acts as a graft-transmissible signal that can inhibit bud growth (Brewer et al. 2009). Other genes in the same signaling pathway have also been discovered. These

include *RMS5*, *MAX3*, and *DAD3* encoding another CCD that appears to perform a redundant role with *RMS1*, *MAX4*, and *DAD1*. A cytochrome P450 enzyme encoded by *MAX1* further modifies the product of the CCD proteins. No functional orthologue of *MAX1* has been identified in petunia or pea at the time of this writing. Likewise, there is no obvious arabidopsis orthologue of *RMS2* or *RMS3*. An F-box containing protein (*MAX2*, *RMS4*, and possibly *DAD2*) perceives the graft-transmissible compound produced by *RMS1*, *MAX4*, and *DAD3*, and is repressed. F-box proteins are components of protein complexes that ubiquitinate specific proteins, thus tagging them for degradation by the 26S proteasome. Several hormone signaling systems, including auxin and gibberellic acid (GA), act through similar F-box proteins. *MAX2* was originally identified in a screen for genes involved in senescence, and thus might function in multiple physiological processes and possibly provide the link responsible for crosstalk between senescence and auxin signaling. It is also worth noting that cytokinin represses senescence (Gan and Amasino 1995). Thus, increased cytokinin levels could regulate *MAX2* expression or function.

MAX mutants actually have more auxin transport in the transpiration stream, and also over-express *PIN1* and other components of the polar auxin transport machinery. This surprising observation suggests the possibility that auxin or some auxin-induced growth inhibitor do not directly inhibit bud outgrowth, but instead inhibit the inability to export auxin, thus inhibiting bud outgrowth (Ongaro and Leyser 2008). This hypothesis suggests that, for bud outgrowth to occur, the bud must be able to export auxin. When the plant is intact, the apical bud(s) saturate the capacity for basipetal auxin transport, thus preventing the axillary buds from exporting auxin themselves, and the *MAX*/*RMS*/*DAD* signaling system detects or regulates the saturation level of auxin transport.

Once the basipetal transport of auxin is disrupted, and the *MAX*/*RMS*/*DAD* pathway induces bud growth, numerous signaling pathways are activated. Growth induction following loss of paradormancy in several model systems was monitored using microarray analysis. Horvath et al. (2005a), working with the perennial weed leafy spurge (*Euphorbia esula*), published the first report of such experiments. Loss of apical dominance resulted in reduced expression of genes involved in flavanoid biosynthesis, as well as in other physiological processes including cell division. Flavanoids have been implicated in reducing auxin transport though inhibition of the auxin transporter *PIN1* (Lazar and Goodman 2006). Thus, the reduction in flavanoids biosynthesis might be required for auxin transport out of the bud. Interestingly, genes involved in cell division, such as *CYCLIN D3* and various histone-encoding genes, are up-regulated following loss of apical dominance (Horvath et al. 2005a). Additionally, several suspected GA-regulated genes are also induced (Horvath et al. 2005a). Cytokinin and GA both impact cell division and cell growth (reviewed in Horvath et al. 2003). Thus, cytokinin and GA production/perception induced by loss of basipetal auxin transport might directly play a role in enhancing bud outgrowth. Experiments on gene expression during paradormancy release in leafy spurge implicated GA in induction of cell cycle genes (Horvath et al. 2002).

4.2.3 Other Factors Regulating Bud Outgrowth

Regulation of bud outgrowth is also impacted by signals other than auxin, or which only indirectly control auxin responses. For example, light impacts bud outgrowth (Snow 1937; Horvath 1999). Similarly, sugars produced in photosynthesizing leaves are capable of inhibiting axillary outgrowth in leafy spurge (Horvath 1999; Chao et al. 2006). In leafy spurge, two signals (sugar and auxin) regulate axillary bud outgrowth through separate mechanisms (Horvath 1999). Sugars directly impact an early phase transition of the cell cycle (G1 to S phase) in adventitious buds of leafy spurge, whereas auxin appeared to impact cell division later in the cycle (Horvath et al. 2002). Nutrients also impact bud outgrowth, but it is not clear whether this is due to altered perception or transport of hormones, regulation of other signaling responses that indirectly affect hormone signaling, or an impact on downstream signals also regulated by these hormones (Cline 1991).

4.3 Regulation of Endodormancy

Endodormancy is a cyclical phenomenon common to many perennial plants. Endodormancy is maintained by signals internal to the buds, which prevent growth even under growth-conducive conditions. Endodormancy in buds often manifests itself immediately prior to seasonal changes that bring about extremes in temperature or moisture that can damage the plant (i.e., during the fall in temperate climates, or immediately prior to the dry season in subtropical zones). Most perennial plants are capable of producing vegetative propagules (shoot buds) and/or protecting the apical meristems that will serve as a source of new growth following periods of environmental extremes that might otherwise result in plant death. Specific physiological processes both inhibit growth and initiate developmental programs that protect buds from damaging environmental conditions, thus allowing the plants to maintain a perennial growth habit. These processes are initiated by environmental signals, primarily short day lengths and/or cold temperatures. It takes little imagination to consider the problems that a perennial might face if, following the loss of the shoot apices to a frost early in the fall, it immediately mobilizes its reserves and initiates outgrowth of axillary or adventitious buds. The resulting shoots would certainly be doomed by subsequent and more prevalent frosts later in the fall.

4.3.1 Hormones in Endodormancy Induction

As with paradormancy, plant hormones play a significant role in endodormancy induction (Fig. 4.2). Historically, ethylene was the first plant hormone associated

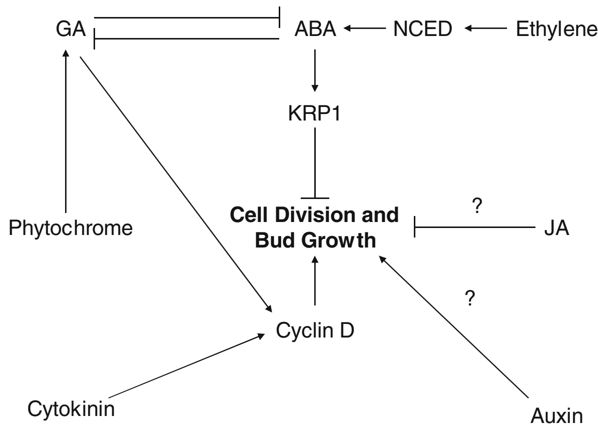

Fig. 4.2 Diagram showing action and interaction of plant hormones on cell division and growth in buds. GA, gibberellic acid; ABA, abscisic acid; JA, jasmonic acid; KRP1, KIP RELATED PROTEIN 1; NCED, 9-CIS-EPOXYCAROTENOID DIOXYGENASE

with seasonal transitions in tree species. In the early 1900s, some astute physiologists noted that trees around gas lamps tended to display developmental responses (leaf loss) normally associated with the onset of fall (e.g., Abeles 1973). Studies have since demonstrated that ethylene is required for bud set. However, these same studies indicated that lack of ethylene responsiveness does not prevent growth cessation in response to short day conditions in poplars (*Populus* sp.; Ruonala et al. 2006). In several plant systems, ethylene levels peak early in the transition to endodormancy, and then drop back to control levels (Suttle 1998; Ruttink et al. 2007). However, this does not imply that ethylene plays no role in seasonal bud growth cessation.

Ethylene induction may initiate a physiological chain of events that lead to growth cessation and dormancy. Ethylene induces *9-CIS-EPOXYCAROTENOID DIOXYGENASE* (*NCED*) in citrus (Rodrigo et al. 2006). NCEDs are proteins required for ABA biosynthesis (Seo and Koshiba 2002). ABA is known to inhibit cell division (and thus growth) by inducing the expression of *ICK1* (also known as *KRP1*), an inhibitor of cyclin-dependant kinases (Wang et al. 1998). ABA also antagonizes GA, and GA accumulation is often associated with plant cell elongation and cell division (Francis and Sorrell 2001). *PHYTOCHROME A* (*PHYA*) expression has a positive impact on several GA biosynthetic genes (Eriksson 2000), and over-expression of *PHYA* inhibits growth cessation and endodormancy (Böhlenius et al. 2006; Ruonala et al. 2008). Cytokinin also plays an opposite role to ethylene by enhancing cell division and preventing senescence (Francis and Sorrell 2001). Thus, it is not surprising that processes associated with cytokinin responses are down-regulated upon endodormancy induction (Rohde et al. 2000).

Interestingly, although auxin is not normally associated with endodormancy, microarray analyses from several different systems have implicated auxin signaling and metabolism during endodormancy induction (Anderson et al. 2005; Ruttink et al. 2007; Horvath et al. 2008). However, the role of auxin in endodormancy is currently unknown. Likewise, endodormancy-inducing conditions may alter jasmonic acid (JA) responses. One hypothesis is that JA induces storage proteins (Druart et al. 2007; Horvath et al. 2008). These storage proteins are needed for buds to survive the dormant state, and renew their growth once growth-conducive conditions return.

4.3.2 Metabolism, Transport, and Cell-Cell Communication Are Altered During Endodormancy

Besides hormonal changes, traditional and transcriptomic-based studies show endodormancy alters other physiological processes. Perhaps the most intriguing is the observation that glycolysis is up-regulated during endodormancy induction (Druart et al. 2007; Keilin et al. 2007; Horvath et al. 2008). The fact that genes encoding proteins required for the TCA cycle do not increase provides some suggestion that endodormant buds undergo oxygen deprivation (Horvath et al. 2008). Keilin et al. (2007) hypothesize that oxidative plays a role in dormancy release, similar to that caused by some dormancy-releasing compounds such a hydrogen cyanide. Another hypothesis developed from the observations is that up-regulation of glycolysis is required for production of sugar alcohols and membrane components needed for cold acclimation. Indeed, glycolytic enzymes are also induced during cold acclimation of *Rhododendron catawbiense* (Wei et al. 2005).

Another physiological process that plays a role in endodormancy induction and maintenance is intercellular transport and communication (Rinne and van der Schoot 1998). In order to grow and develop normally, cells within the bud need to communicate with each other. Yet, as buds transition into endodormancy, 1,3-β-D-glucans accumulate in the plasmodesmata and block cell-cell communication. Endodormancy-releasing conditions induce glucanases that degrade 1,3-β-D-glucans (Rinne et al. 2001). Rinne et al. (2001) hypothesized that these blockages are, at least in part, responsible for inhibition of bud growth following endodormancy induction. Interestingly, endodormancy induction in several plant species induces a large number of genes involved in transport functions (Horvath et al. 2008). Many of these genes are involved in sugar transport, as well as intracellular amino acid transport between the cytoplasm and the mitochondria. Horvath et al. (2008) hypothesized that these transport functions were involved in solute accumulation needed for cold hardening processes. Since transport of sugars and amino acids does not require functioning plasmodesmata or intercellular communication, these two hypotheses are not mutually exclusive.

4.3.3 Regulation of Endodormancy by Environmental and Physiological Signals

Temperature and light quality initiate many of the physiological responses associated with dormancy, including growth cessation, bud set, and cold hardening (During and Bachmann 1975; Rinne et al. 1994; Olsen et al. 1997; Chen et al. 2002). These signals act on well-conserved growth and development genes that are also reasonably well characterized in a few model systems such as *Arabidopsis* (Rohde et al. 1999; Horvath et al. 2003). The mechanisms by which these signals bring about endodormancy are only beginning to be unraveled (Fig. 4.3). Interestingly, although low temperatures can enhance endodormancy induction, extended cold temperatures also bring buds out of endodormancy, and revive their growth competence. One interesting observation is that the same extended cold treatment required for dormancy release also makes buds of many perennial species flowering competent as well (Chouard 1960; Metzger 1996; Horvath et al. 2003; Rohde and Bhalerao 2007). This observation has led to the hypothesis that mechanisms

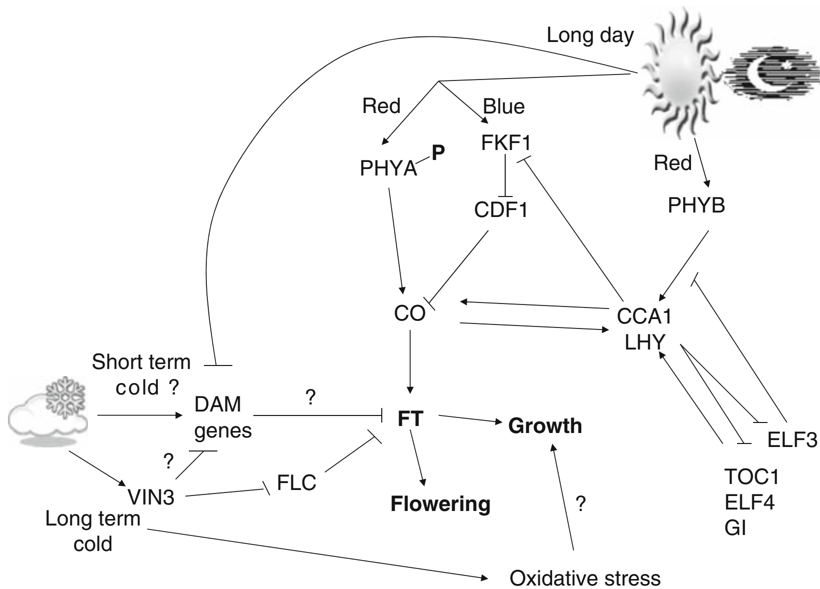

Fig. 4.3 Schematic of signaling pathways and genes involved in regulating both flowering and bud growth. GI, GIGANTEA; ELF4, EARLY FLOWERING4; TOC1, TIMING OF CAB EXPRESSION1; ELF3, EARLY FLOWERING3; LHY, ELONGATED HYPOCOTYL; CCA1, CIRCADIAN CLOCK ASSOCIATED1; PHYB, PHYTOCHROME B; FKF1, FLAVIN-BINDING KELCH REPEAT F BOX1; CDF1, CYCLING DOF FACTOR 1; PHYA, PHYTOCHROME A; CO, CONSTANS; FT, FLOWERING LOCUS T; DAM, DORMANCY ASSOCIATED MADS-BOX; VIN3, VERNALIZATION INSENSITIVE3

regulating flowering and vernalization may also play a role in endodormancy induction and release.

Decreasing day length induces growth cessation and bud set in temperate trees such as poplar and birch (*Betula* sp.). Bud set is a process during which leaf primordia are modified to form hard scales that protect the meristem from harsh winter conditions. Trees from more northern latitudes cease growth and set buds in much longer day lengths than do trees from more southern latitudes. Sensing of day length has been well studied in model systems such as arabidopsis, generally in relation to flowering responses. In arabidopsis, many of the genes implicated in day-length sensing are components of the circadian clock (McClung 2006).

Briefly, the ratio of red light to far-red light is different during dusk and dawn as compared to the middle of the day. PHYA and PHYB sense the ratio of red to far-red light. These proteins then act either alone or together with CRYPTOCHROME 1 (CRY1, a blue light receptor) to regulate the expression of *CONSTANS* (*CO*; Fig. 4.2). The regulation of *CO* by these photoreceptors occurs via the action of several intermediate signaling components such as FLAVIN BINDING KELCH REPEAT F-BOX1 (FKF1), CIRCADIAN CLOCK ASSOCIATED1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY), CYCLING DOF FACTOR 1 (CDF1), and EARLY FLOWERING 3 and 4 (ELF3/4), all of which are major constituents of the circadian clock mechanism. CO regulates the expression of *FLOWERING LOCUS T* (*FT*), a major floral regulator. Not surprisingly, seasonal transitions or shortened day lengths that impact endodormancy induction involve many of the genes differentially regulated during circadian responses. Microarray analysis has characterized transcriptome changes associated with transitions into and out of endodormancy in several different perennial species (Druart et al. 2007; Mazzitelli et al. 2007; Ruttink et al. 2007; Campbell et al. 2008; Horvath et al. 2008). In all of these experiments, multiple genes involved in circadian responses were identified as differentially expressed (Horvath et al. 2008).

Eriksson (2007) established that reduced expression of *PHYA* results in down-regulation of *CO* that leads to growth cessation and bud set in poplar. Ruonala et al. (2008) further determined that *PHYA* over-expression inhibited endodormancy induction. The exact mechanisms by which this occurs are being examined. Interestingly, transgenic trees over-expressing *PHYA* also have increased levels of *FT1* in mature leaves (Böhlenius et al. 2006). Expression of two other *FT*-like genes (*FT2* and *CENTRORADIALIS-LIKE1*) initially decreases in source leaves (younger leaves close to the apical bud) in response to short day in poplar, but then rapidly recovers to near-control levels in *PHYA* over-expressing lines (Ruonala et al. 2008). Such transgenic tress not only flower earlier than wild type, but also require much shorter day lengths before ceasing growth, and thus mimic trees from more southern latitudes (Böhlenius et al. 2006). This appears to be a direct response to expression of *FT*. Transgenic trees over- or under-expressing *FT1* show altered day-length thresholds required for growth cessation and bud set (Böhlenius et al. 2006). Thus, it appears that the *FT1* acts not only as a positive regulator of flowering, but also as a negative regulator of seasonal growth cessation and bud set. This phenomenon is likely not limited to poplar, since expression of *FT*-like genes is also

down-regulated in adventitious buds of leafy spurge during and following endo-dormancy (Horvath et al. 2008).

In arabidopsis, a number of specific MADS-box transcription factors, notably FLOWERING LOCUS C (FLC), and SHORT VEGETATIVE PHASE (SVP), regulate *FT* expression. Similar transcription factors also regulate bud dormancy in several perennial species (Bielenberg et al. 2008; Horvath et al. 2008). Comparison of microarray studies from different species identified a number of *DORMANCY ASSOCIATED MADS-box* (*DAM*) genes, one of which is induced when buds enter endodormancy, and is then down-regulated when buds transition from endodormancy into ecodormancy (Horvath et al. 2008). The *EVERGROWING* locus from peach has provided functional confirmation that *DAM* genes are involved in the dormancy process (Bielenberg et al. 2008). Peach varieties that contain the *evergrowing* mutation do not cease growth or set buds during dormancy-inducing short day treatments (Diaz 1974). Sequencing of the *evergrowing* locus identified the mutation as a deletion in a series of *DAM* genes (Bielenberg et al. 2004). *DAM* genes are similar to *SVP*, and SVP negatively regulates *FT* in arabidopsis (Michaels et al. 2003; Lee et al., 2007). *FT* regulates seasonal growth cessation and bud set (Böhlenius et al. 2006). *DAM* gene structure and expression patterns are conserved (Horvath, unpublished data). Thus, it will be surprising if *DAM* genes are not involved in regulating growth cessation and bud set by negatively regulating *FT* or *FT*-like genes such as *CENL1* in most perennials.

Light-regulated expression of *FT* is likely only a portion of the mechanism regulating endodormancy transitions. *FT* expression is down-regulated upon endodormancy induction, but it stays low even after extended periods of cold temperatures have released endodormancy. Likewise, some *DAM* genes are expressed after buds have transitioned from endodormancy to ecodormancy in at least two perennial species (Horvath et al. 2008). Therefore, although endodormancy induction may depend, at least in part, on induction of *DAM* genes and repression of *FT*, dormancy release appears to work through a different mechanism. Studies are underway in the laboratory of Dr. Ove Nilsson to determine when *FT* expression resumes following endodormancy release, and what effects induction of *FT* might have on already dormant buds.

4.3.4 Endodormancy Release

As noted above, endodormancy release and renewed growth competence of buds require extended periods of cold temperatures. The molecular mechanism causing endodormancy release is unknown. However, like endodormancy induction, there are parallels to floral regulation. This correlation is primarily due to the observations that the same environmental signals appear to regulate renewal of both growth and floral competence (namely, day length and extended cold temperatures), and because both phenomena appear to require some epigenetic memory component.

Although the mechanisms through which extended cold temperatures release endodormancy are not known, in wheat and arabidopsis the mechanisms by which extended cold temperatures regulate floral competence are well characterized. In arabidopsis, a *MADS-box* gene called *FLOWERING LOCUS* C (*FLC*) is a key floral regulator (Borner et al. 2000). FLC suppresses *FT* expression (Samach et al. 2000). However, extended cold temperatures cause modification of the chromatin structure around the promoter of *FLC*, and epigenetically turn *FLC* off (Sung and Amasino 2004). Three genes—*VERNALIZATION INSENSITIVE 3* (*VIN3*), and *VERNALIZATION 1* and *2* (*VRN1* and *VRN2*)—are involved in modifying the chromatin structure of the *FLC* promoter (Sung and Amasino 2004). As noted above, an additional MADS-box protein, SVP, works together with FLC to regulate *FT* expression (Lee et al. 2007). *AGL24*, another MADS-box gene closely related to *SVP*, which induces flowering, is up-regulated by extended cold (Michaels et al. 2003). In wheat, altered expression of an orthologue of *FT* designated *VRN3* is regulated by a *CO*-like gene designated *VRN2*, and an *MADS-box* transcription factor named *VRN1*. It is suspected that *VRN1* of wheat is epigenetically regulated (Dennis and Peacock 2007), but since *VRN1* is a floral inducer, rather than a floral repressor, it is unlikely to be a functional analogue of *FLC*. Given the similarity between vernalization and endodormancy release, Horvath et al. (2003) hypothesized that chromatin remodeling proteins also regulate endodormancy release during extended cold temperatures.

Besides the likely conservation of response mechanisms between vernalization and endodormancy release, there is additional evidence that epigenetic modifications are involved in endodormancy induction and maintenance. Results of most transcriptome analyses reported so far have identified several chromatin remodeling genes as being differentially expressed. Specifically, these include a chromatin modifying SWI2/SNF2-like protein that is down-regulated during dormancy release in potato and leafy spurge. A related gene is up-regulated in poplar during dormancy induction, and a different SNF-like protein is up-regulated following a dormancy-breaking treatment of hydrogen cyanide in grape (*Vitis* sp.; Or et al. 2000; Ruttink et al. 2007; Campbell et al. 2008; Horvath et al. 2008). Likewise, Law and Suttle (2004) observed general chromatin modifications in potato following conditions leading to dormancy release.

Besides alterations of chromatin involving perception of extended cold, another hypothesis put forth is that perception of oxidative stress is the primary mechanism for endodormancy release. This hypothesis essentially derives from observations that application of chemicals such as hydrogen cyanide and heat shock can also release buds from endodormancy (Shulman et al. 1986; Or et al. 2000). These treatments, along with cold temperatures, induce oxidative stress responses in buds. In some cases, brief and transient bursts of oxidative stress appear sufficient to break endodormancy (Or et al. 2002), thus negating the requirement for extended periods of cold. The mechanism through which oxidative stress releases buds is as yet unknown. However, some manipulation of calcium signaling appears to impact the effects of oxidative stress on endodormancy release (Pang et al. 2007). Likewise, it was noted that catalases are significantly down-regulated prior to dormancy

release, and upon treatment with hydrogen cyanide (Or et al. 2002). Or et al. (2000) speculated that this oxidative stress could lead to an altered AMP to ATP ratio, which could be sensed via the SNF signaling pathway. If this is the case, then there might be a linkage between oxidative stress and chromatin remodeling.

Several interesting growth-regulating genes were differentially expressed during the transition from endodormancy to ecodormancy in leafy spurge (Horvath et al. 2008). These included *RETINOBLASTOMA*-like (*RB*-like) protein, *GROWTH REGULATING FACTOR5* (*GRF5*), and *ARABIDOPSIS MEI2-LIKE1* (*AML1*). RB sequesters several growth-promoting transcription factors, until CDKA phosphorylates it. RB also plays a direct role in chromatin modification (Shen 2002). GRF5 and AML1 are both suspected to be positive regulators of growth (Anderson and Hanson 2005; Horiguchi et al. 2005; Kaur et al. 2006). Thus, it is intriguing that they are up-regulated during endodormancy release at a time when other cell cycle regulators are generally down-regulated (Horvath et al. 2008).

4.4 Ecodormancy

Once buds have received sufficient cold to break endodormancy, in most temperate climates buds remain non-growing until temperature and moisture reach levels capable of sustaining growth. Until that time, these growth-competent buds are considered to be ecodormant—that is, adverse environmental conditions simply keep them from growing. Cold and drought prevent growth through as yet unknown mechanisms. However, one hypothesis suggests that part of the mechanisms involve the induction and maintenance of ABA levels within the buds (Horvath et al. 2003). ABA accumulates under both drought and cold conditions experienced by ecodormant buds. Also, ABA concentrations are well associated with bud dormancy maintenance in numerous perennial species (reviewed in Horvath et al. 2003). As noted above, ABA up-regulates expression of cell cycle inhibitors. Also, ABA inhibits expression of *CYCD3* and *CDKB* (De Smet et al. 2003), two key cell cycle regulators (see below). Thus, high ABA levels may simply maintain ecodormancy by inhibition of cell division.

4.5 Regulation of Cell Division and Development Is Important for All Forms of Dormancy

Buds perceive environmental and physiological signals regulating all forms of dormancy, and these signals generate further signaling cascades. These signaling cascades often include various transcription factors and plant hormones. However, at some point, all of these signals must impact the bud meristem's ability to grow and develop. Nearly all transcriptome analyses of dormancy have identified key cell cycle genes as being repressed when buds become dormant, and these same genes

are up-regulated upon growth induction following dormancy release. Thus, it seems likely that cell cycle regulation is one of the key physiological processes altered by dormancy signals.

There are numerous good reviews of the plant cell cycle, and thus this review will provide only a brief outline of the components, and their relationship to various dormancy signals. The first cell cycle genes induced when plant or animal cells shift from dormancy to growth are D-class cyclins (*CYCD*). There are several different *CYCD* genes in plants (Dewitte and Murray 2003). *CYCD-3* is perhaps the most interesting. Sugar, GA, and cytokinin regulate *CYCD-3*, and all three of these compounds regulate bud growth (Hu et al. 2000; Horvath et al. 2002; Chao et al. 2006).

Indeed, *CYCD-3* is up-regulated soon after loss of paradormancy, and down-regulated following endodormancy in leafy spurge (Horvath et al. 2005b, 2008). CYCD-3 interacts with CDKA to phosphorylate the RETINOBLASTOMA (RB) protein, and also interacts with CDKB to initiate mitosis (Shen 2002). Several CDK proteins accumulate in potato buds following dormancy release (Campbell et al. 1996). Although *RB* is not differentially expressed in some systems, it may be differentially phosphorylated following dormancy transition in poplar (Espinosa-Ruiz et al. 2004). Likewise, several *RB*-like genes are differentially expressed during dormancy transitions in leafy spurge (Horvath et al. 2008). Interestingly, the role of cell cycle regulation during endodormancy is somewhat species-specific. Cell cycle genes do not appear to be significantly down-regulated in crown buds of leafy spurge until the buds shift from endodormancy to ecodormancy (Horvath et al. 2008). In poplar, by contrast, they are down-regulated as the buds enter endodormancy (Espinosa-Ruiz et al. 2004; Druart et al. 2007). Thus, although cell cycle is commonly differentially regulated during dormancy transitions in perennials, they may be regulated by different signals.

In addition to regulating the cell cycle, GA, cytokinin, and CYCD also impact meristem development through regulation of genes such as *SHOOTMERISTEMLESS* (*STM*; Meijer and Murray 2001). STM is a homeobox containing transcription factor required for maintaining the reservoir of undifferentiated cells in the meristem from which the various plant organs are derived (Hay et al. 2004). Upon release of buds from paradormancy in leafy spurge, *STM* is induced (Varanasi et al. 2008). Several related homeobox transcription factors are differentially expressed during endodormancy induction in poplar (Ruttink et al. 2007). Interestingly, STM protein diffuses from the central region of the meristem to more peripheral cell layers, where it promotes cell division and regulates leaf development (Kurata et al. 2005). Thus, it is possible that the restriction of the plasmodesmata interrupts movement of STM and other diffusible developmental signals, and thereby blocks bud development and growth.

Other transcription factors are also generally associated with dormancy transitions. Studies in poplar identified 12 different transcription factors that were also associated with dormancy to growth transitions in seeds and cambial meristems (Ruttink et al. 2007). Horvath et al. (2008) noted that *AGAMOUS LIKE63* (*AGL63* (*JOINTLESS*)), *BLIND*, *INDUCER OF CBF EXPRESSION* (*ICE1*), *HOMEOBOX3*

(*HOX3*), 2 *MYB*, 2 *MYC*, *HEXAMER BINDING PROTEIN-1b* (*HBP-1b*), *HOX 4*, *WRKY* (A1244, 30, 53), and six different zinc finger-encoding genes were differentially expressed in at least three different species in response to conditions impacting bud dormancy. The roles that these genes play in modifying the physiology of buds during dormancy transitions remain to be illuminated, but some transcription factors such as *AGL63* and *BLIND* play well-characterized roles in meristem development. The conserved differential expression of these transcription factors in various organs and species undergoing dormancy transitions suggests that they play important roles.

4.6 Future Perspectives

The advent of genomics offers exciting new tools needed to answer questions raised by years of good physiological studies on various aspects of bud dormancy. The sequencing of numerous plant genomes, and the increasing use of powerful transcriptomic analyses on crops, weeds, and model species are opening the opportunity for comparative studies that are certain to provide insights into dormancy regulation and evolution. The identification of *DAM* genes through such comparative analyses serves as a prime example of how these approaches can rapidly provide insight into mechanisms regulating bud dormancy. Indeed, the availability of the genomic sequence surrounding *DAM* genes has pinpointed likely orthologues in several related species (Horvath, unpublished data). Likewise, phylogenetic footprinting using readily available sequences from promoters of differentially expressed genes from multiple species will speed up the identification of factors and components of dormancy-regulating processes. The ability to map-base clone dormancy genes in non-model species such as peach, as demonstrated by the characterization of the *EVERGROWING* locus, points toward additional exciting possibilities for the identification of key regulatory processes that impact bud dormancy. Quantitative trait loci that impact dormancy have been identified in poplar and dogwood (Chen et al. 2002; Svendesen et al. 2007), and the sequencing of the poplar genome makes identification of the underlying genes likely in the near future. Likewise, the ability to genetically manipulate model perennials such as poplar will greatly facilitate testing the functionality of genes identified by transcriptomic and comparative means. Thus, it seems likely that, by the time this book is published, notable advances will have been made, and our understanding of how bud growth is regulated will be considerably greater than it is at the time of writing.

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Chapter 5
Root Development

L. Jansen, B. De Rybel, V. Vassileva, and T. Beeckman

5.1 Introduction

One of the main problems plants encounter, due to their sessile nature, is the complex environment in which they have to thrive, for example, a mixture of solid, gaseous and liquid phases wherein nutrients are unequally distributed. Plants have conquered these difficulties by developing highly adaptive and adequate species-specific root architectures. As a result of evolutionary mechanisms, different types of roots and root systems can be studied today. Although most root systems are formed below ground, in some cases roots can be initiated from aerial parts to give extra support. Plants may also develop storage roots, cluster roots or form root nodules with nitrogen fixing bacteria for nutrient storage and acquisition. Although important for certain species, these types of roots are designated mainly as adventitious roots, which have been reviewed recently (Geiss et al. 2009).

5.2 Plant Root Systems, All But Uniform

5.2.1 Root Types

Angiosperms display a large diversity of root systems that have been classified into different types (Canon 1949). In all cases, a primary root that is initiated during embryogenesis becomes first visible upon germination. This root grows straight

L. Jansen, B. De Rybel, and T. Beeckman
Department of Plant Systems Biology, VIB, Technologiepark 927, 9052 Gent, Belgium
e-mail: tom.beeckman@psb.vib-ugent.be

L. Jansen, B. De Rybel, and T. Beeckman
Department of Molecular Genetics, Ghent University, Technologiepark 927, 9052 Gent, Belgium

V. Vassileva
Academik Metodi Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Academik Georgi Bonchev Street, Building 21, 1113 Sofia, Bulgaria

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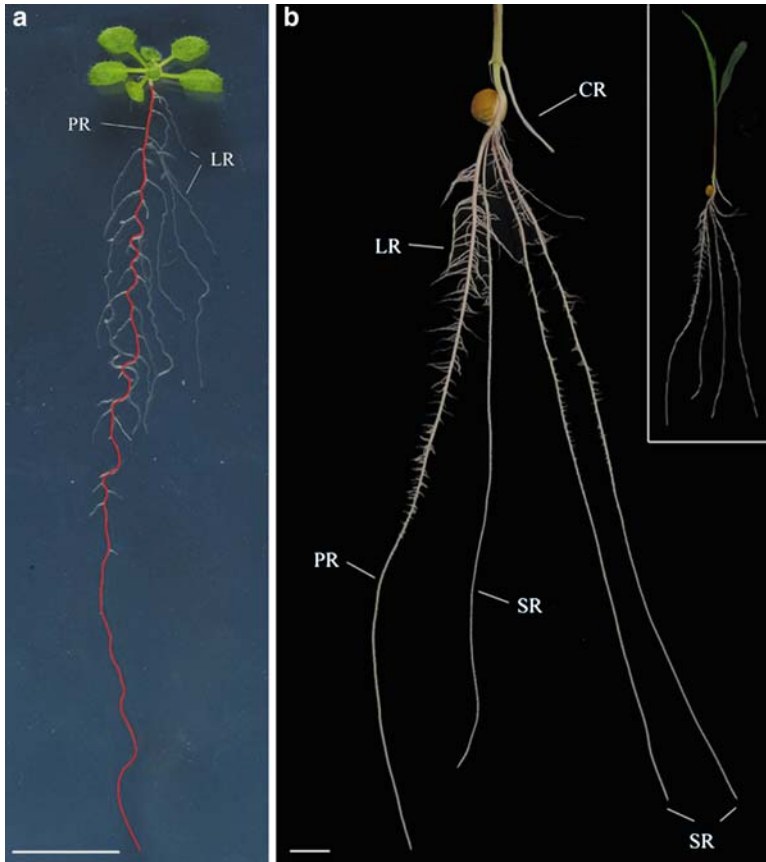

Fig. 5.1 Representation of different root types of *Arabidopsis thaliana* (**a**) and *Zea mays* (**b**). PR, primary root; LR, lateral root; SR, seminal root; CR, crown root. Bar = 1 cm

downwards and screens different layers of the soil for water and nutrients. Lateral branches form all over the primary root, thereby extending the surface area considerably. In cases where the primary root remains functionally important during the lifetime of the plant, a taproot system develops (Fig. 5.1a), which is characteristic of many dicotyledonous plants, including Arabidopsis. In most monocotyledonous plants, the primary root system is only important during early development of the plant (Feix et al. 2002). In these species, an extensive secondary root system supports the plant during later stages, resulting in a fibrous root system. In maize, the embryonic root system is extended with seminal roots, which become visible 3 days after germination from the scutellar node (Fig. 5.1b). At later stages during development, adventitious roots also emerge from stem-nodes beneath and above soil level, which are named crown and brace roots respectively (Hochholdinger et al. 2004). Due to the formation of lateral branches on all of these root types, the total length and absorptive area of a root system are extended exponentially.

5.2.2 Genetic Variation in Root Architecture

The major outline of the root system is genetically determined within species. However, characteristics of root architecture can also differ between genotypes. In maize, for instance, the number of brace roots per node, as well as their colour and diameter are variable between different inbred lines, even when the same growth conditions are applied (Fig. 5.2). This genetic variation can be used as a basis for the identification of quantitative trait loci (QTLs) that can lead to the discovery of genes involved in basic processes regulating root architecture. Using genetic variation between different Arabidopsis accessions, several QTLs have been identified as responsible for important aspects of root architecture, such as primary root length, lateral root density and lateral root length (Mouchel et al. 2004; Loudet et al. 2005). Primary root length, for instance, was shown to be under control of *BREVIX RADIX*, a member of a novel plant specific gene family with unknown function (Mouchel et al. 2004, 2006). Interestingly, by studying different developmental stages in rice, it was shown that most QTLs for root traits were selectively expressed at different stages (Qu et al. 2008). Also, the response of plants to their environment by the adaptation of their root system can vary between genotypes. This has led to the identification of QTLs responsible for root traits under phosphate starvation and drought stress in Arabidopsis and maize (Reymond et al. 2006; Zhu et al. 2006; Landi et al. 2007).

5.2.3 Hormonal Control of Root Architecture

The effect of different plant hormones is a major topic in the study of root formation. Several hormones are known to influence root architecture, usually

Fig. 5.2 Brace roots of three different maize inbred lines demonstrating genotypic variation within plant species. Bar=5 cm

through the inhibition or induction of lateral roots. Auxin is a major player in shaping root systems by regulating growth of primary and lateral roots. Specifically, normal auxin transport and signalling are indispensable for the initiation and development of lateral roots (Reed et al. 1998; Fukaki et al. 2002; see Chap. 6.3). An interplay between auxin and ethylene in root growth was described in pea in the 1970s (Chadwick and Burg 1970). Recently, this interaction has been studied in more detail. It includes low concentrations of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), which induce auxin biosynthesis, while high concentrations were thought to increase auxin concentrations up to levels inhibitory for root growth and lateral root development (Ivanchenko 2008). Furthermore, high ACC concentrations also increased the capacity of auxin transport by regulating the transcription of auxin transport components. Cytokinin and auxin have antagonistic effects on root formation. By influencing auxin transport and homeostasis, cytokinin inhibits lateral root formation (Laplaze et al. 2007). Auxin can, in turn, directly downregulate cytokinin biosynthesis (Nordstrom et al. 2006). In rice, cytokinin was also shown to inhibit lateral root initiation, but stimulate lateral root elongation (Rani Debi et al. 2005). Recently, abscisic acid (ABA) was also identified to play a role during root development. Although its exact role during lateral root initiation is not clear, it has been suggested that auxin and ABA act antagonistically during lateral root initiation (De Smet et al. 2006). In later stages during lateral root formation, ABA can inhibit the growth of primordia prior to the activation of the lateral root primordia or shortly after emergence (De Smet et al. 2003). Jasmonic acid has been shown to induce lateral root formation in rice, but knowledge has been limited on the mechanisms. As there is no correlation between the number and distribution of lateral roots induced by auxin and jasmonic acid, both hormones are thought to act independently (Wang et al. 2002). Nevertheless, results of these studies indicate the pivotal role of hormonal cross-talk in controlling the final architecture of the root systems. Future research in this area will be essential to disentangle the underlying mechanisms.

5.2.4 Environmental Factors Influencing Root Architecture

The environment also greatly influences root architecture, as plants need to optimize their root system for nutrient acquisition. For example, plants that experience nitrate deficiency try to overcome this situation by accelerating root growth and increasing the number of lateral branches, thus increasing their foraging capacity (Tranbarger et al. 2003). A local high nitrate concentration induces initiation and elongation of lateral roots in the nitrate-rich zone, while primary and lateral root growth is suppressed outside these areas (Zhang et al. 1999; Linkohr et al. 2002). In maize and barley, lateral root proliferation occurs in regions with higher nitrate availability, although no effect on primary root growth was reported previously (Drew 1975; Granato and Raper 1989). In contrast, an overall high nitrate concentration in the soil inhibits lateral root growth (Zhang and Forde 1998). The local and general responses are mediated by different

pathways and act at different stages during lateral root development (Zhang et al. 1999). The localized stimulatory effect is triggered by a high concentration of nitrate sensed at the tip of mature lateral roots through the amount of nitrate that is taken up (Zhang and Forde 1998; Remans et al. 2006a, b). Three nitrate transporters, encoded by *NRT1.1*, *NRT1.2* and *NRT2.1*, have been identified in Arabidopsis, which may account for a major part of the nitrate acquisition in plants (Cerezo et al. 2001; Munos et al. 2004). NRT1.1 is the main transport system, mediating low- and high-affinity transport through phosphorylation, and may also serve as a nitrate sensor (Liu and Tsay 2003; Munos et al. 2004; Remans et al. 2006a). Moreover, through a yet unknown mechanism, NRT1.1 influences the expression of *ANR1*, a MADS-box transcription factor expressed in roots, triggering lateral root elongation in nitrate-rich areas (Remans et al. 2006a). NRT2.1 also plays a role in root system architectural changes in response to nitrate availability. Although its exact role is not clear, NRT2.1 seems to influence both lateral root initiation and emergence independently of its function as a nitrate transporter (Little et al. 2005; Remans et al. 2006b). In contrast to the stimulatory effect, the inhibitory response at high nitrate concentration is systemic and depends on a signal from the shoot, based on the amount of nitrate absorbed by the whole root system (Zhang and Forde 1998). This response is independent of *ANR1* and inhibits lateral root growth before activation of the newly formed primordium.

Phosphate deprivation has a major influence on root architecture in Arabidopsis by reducing primary root elongation, while growth of lateral roots and root hairs is induced, increasing the foraging and uptake capacity of the root system (Lopez-Bucio et al. 2002). In contrast, lateral root emergence is arrested at a high phosphate concentration. In most maize inbred lines, the number and length of lateral roots are favoured by phosphate shortage, as well as the number and length of seminal roots (Zhu et al. 2005, 2006).

Through mutant and QTL analysis in Arabidopsis, some factors have been identified that play a role in phosphate sensing and/or response. For example, *pdr2* is hypersensitive to low phosphate, strongly inhibiting primary root growth and initiating a greater number of lateral roots (Ticconi et al. 2004; Reymond et al. 2006; Svistoonoff et al. 2007). PDR2 is probably part of a signalling pathway that links to the sensing of low phosphate and the reduction of activity of meristematic cells (Ticconi et al. 2004). LPR1 (low phosphate root1), originally identified as QTL, is also involved in root growth arrest upon low phosphate sensing (Reymond et al. 2006; Svistoonoff et al. 2007). *LPR1* encodes a multicopper oxidase and is expressed in the primary root meristem and root cap cells. It has been suggested that LPR1 proteins in the root cap can influence meristem activity, modifying the activity and/or distribution of a hormone-like compound (Svistoonoff et al. 2007). Auxin may be a candidate, as plants grown on low phosphate appear to be more sensitive to the effects of auxin than those grown under high phosphate conditions (Lopez-Bucio et al. 2002). The inhibitory effect of low phosphate on primary root growth seems to be independent of auxin transport, as impairing auxin transport results in a reduced number of lateral roots in phosphate-deficient plants (Lopez-Bucio et al. 2002, 2005).

Transcriptome analysis revealed several transcription factors involved in root response to phosphate deprivation. WRKY75, a general regulator of lateral root and root hair growth, is strongly induced upon low phosphate conditions. It may be involved in global phosphate starvation response by regulating phosphate-transporters and phosphatases, thus facilitating phosphate acquisition (Devaiah et al. 2007a). *ZAT6* (zinc finger of Arabidopsis 6) shows a similar expression pattern as *WRKY75*, and both act as repressors of root development, suggesting that they may have mutually synergistic effects (Devaiah et al. 2007a, b). However, ZAT6 does not play a role in root development under non-stress conditions. It may be involved in response to other stresses such as potassium, iron and nitrogen (Devaiah et al. 2007b). With respect to phosphate deprivation, some species belonging to the families Proteaceae and Fabaceae are capable of altering their root architecture dramatically by the production of bunches of short-remaining specialized lateral roots, designated as proteoid roots (Johnson et al. 1996). The proteoid roots in all plant species can be mimicked to some extent by applying high concentrations of auxin, and it is tempting to speculate that phosphate starvation cross-talks to auxin homeostasis or response in these species. Although very similar to normal lateral roots, proteoid roots produce more citrate and malate to mobilize soluble mineral and organic phosphate in the soil, thus increasing the available phosphate (Johnson et al. 1996).

5.3 Patterning During Root Embryogenesis

As discussed above, there is an enormous plasticity of root systems and very diverse root system architectures within species, dependent on natural variation, environmental factors and hormonal control. Despite the great diversity in root system architecture, the patterning events during embryogenesis are strictly conserved within species. Although most research has been performed on *Arabidopsis thaliana*, recent advances in other model species have shown that, even though dicotyledonous and monocotyledonous plants show differences in root system architecture and cellular patterning, most of the genetic pathways involved are relatively well conserved (Hochholdinger and Zimmermann 2008). In view of this, the discussion is focused on patterning events during root embryogenesis in Arabidopsis, and indicates conserved and distinct pathways when appropriate.

5.3.1 Early Embryogenesis Patterning Events

The establishment of the apical–basal axis is one of the earliest events during embryogenesis. It is of vital importance for the determination of which cells will later form the embryonic root. Following fertilisation of the egg cell, the Arabidopsis zygote divides into a small apical cell and a large basal cell (Fig. 5.3).

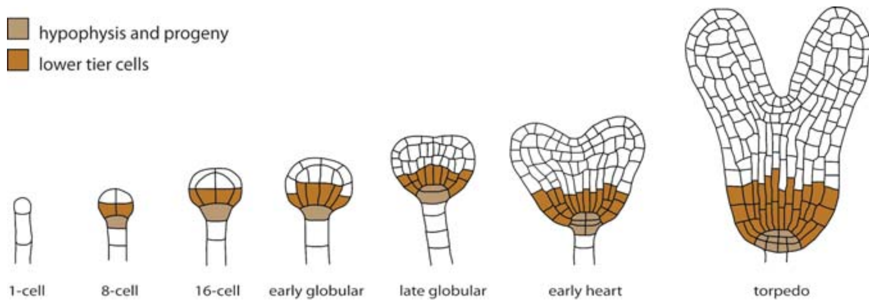

Fig. 5.3 Embryonic development in Arabidopsis thaliana

The apical cell and its daughter cells divide twice longitudinally and once transversely to form a spherical pro-embryo of eight cells. Meanwhile, the basal cell and its derivatives divide transversely to produce the suspensor (Fig. 5.3). The descendants of the uppermost cell of the suspensor, the hypophysis, become integrated in the primary root meristem. At the eight-cell stage, several types of cells are clearly distinguishable, namely the upper tier cells, lower tier cells, hypophysis and suspensor. Both the lower tier cells and the hypophysis contribute to the creation of the root (Fig. 5.3; Dolan et al. 1993). During the subsequent divisions to the 16-cell stage, most of the patterning is established and the apical-basal axis of the embryo is specified, resulting in polarisation of the embryo.

During the early developmental processes, several genes have been identified with differential expression patterns between the apical and the basal cell. The MAPKK kinase gene, *YODA*, has been shown to promote the suspensor cell fate in the basal cell lineages. In loss-of-function mutants, suspensor cells are incorporated into the embryo, whereas there is excessive suspensor growth and suppression of embryo formation in gain-of-function mutants (Lukowitz et al. 2004). Furthermore, the expression domains of *WUSHEL-RELATED HOMEOBOX* genes, *WOX2* and *WOX8*, initially coincide in the zygote and later become restricted specifically in the apical and basal cell respectively (Haecker et al. 2004; Breuninger et al. 2008). *WOX8* expression in the basal cell has been shown to be required for the correct expression of *WOX2* in the apical cell and normal embryo development, suggesting a non-cell autonomous inductive mechanism (Breuninger et al. 2008).

In addition to these specific gene functions, polar auxin transport by members of the PIN family (reviewed in Paponov et al. 2005) has been shown to be a major determinant of the patterning events during embryogenesis (Friml et al. 2003). Prior to the globular stage (Fig. 5.3), auxin is transported upwards through the suspensor to the apical cell by PIN7 located in the apical cell wall of the basal cell. Later, the apical part of embryo at the globular stage begins to produce free auxin. This leads to a switch in polarity, resulting in apical to basal transport. Auxin accumulates in the hypophysis by PIN1- and PIN4-dependent transport, triggering root pole specification (Friml et al. 2003). The WOX2-WOX8 signalling cascade has also been shown to regulate PIN1 expression and the establishment of an auxin maximum in

the pro-embryo (Breuninger et al. 2008). Mutations in multiple members of the PIN family caused severe embryo defects (Friml et al. 2003). Similar phenotypes have been observed after treatment with polar auxin transport inhibitors, such as *N*-1-naphthylphthalamic acid (Hadfi et al. 1998), and by disrupting correct polar PIN protein localization by mutations in genes like the ARF-GEF (guanine-nucleotide exchange factor for ADP-ribosylation factor GTPases) *GNOM* (Liu et al. 1993; Geldner et al. 2003) and phosphatase *PINOID* (Friml et al. 2004). In addition to auxin transport, a correct auxin response is required for hypophysis specification. In both cases, the genes involved appear to be conserved amongst crop species (Sato et al. 2001; Carraro et al. 2006). The central players in auxin signalling specific for early embryogenesis are the auxin response factor (ARF) family represented by MONOPTEROS (MP/ARF5; Hardtke and Berleth 1998) and NON-PHOTOTROPIC HYPOCOTYL4 (NPH4/ARF7; Harper et al. 2000), and their labile repressors of the Aux/IAA family proteins, such as IAA12/BODENLOS (BDL; Hamann et al. 1999) and IAA13 (Weijers et al. 2005).

5.3.2 Establishment of the Primary Root Meristem

Once the apical-basal axis is established and the hypophysis has become specified during early embryogenesis through polar auxin transport and signalling, the primary root meristem needs to be organised in a controlled fashion. Although early patterning genes are essential for proper embryonic root development, the actual establishment of the primary root meristem is initiated from the globular embryo stage onwards with the asymmetrical division of the hypophysis (Fig. 5.3). This division creates a small apical, lens-shaped cell that forms the organising centre of the primary root meristem, designated as the quiescent centre (QC), and a larger basal cell that forms the columella stem cells (Dolan et al. 1993; van den Berg et al. 1998). The QC maintains the undifferentiated state of the surrounding stem cell niche by local signalling (van den Berg et al. 1998; Sabatini et al. 2003). Each of the stem cells surrounding the QC gives rise to one of the specific cell types of the root. The QC and columella stem cells are derived from the hypophysis, whereas the stem cells for the other root tissues are derived from the lower tier (Fig. 5.3, Dolan et al. 1993; van den Berg et al. 1998). The signal determining the developmental fate of these progenitor cells is probably provided by older adjacent cells in the same cell file (van den Berg et al. 1995; Malamy and Benfey 1997). This equilibrium between inhibition of stem cell differentiation by the QC and opposing stimulatory signals from more mature tissues within the cell file determines the root meristem activity.

Evidence from several lines of study suggests that the establishment of a fully functional QC requires the correct expression of two types of transcription factors, namely the GRAS-type *SCARECROW* (*SCR*; Di Laurenzio et al. 1996) and *SHORT ROOT* (*SHR*; Helariutta et al. 2000). The SHR/SCR pathway appears to be strongly conserved in cereals (Lim et al. 2000, 2005; Cui et al. 2007). Furthermore, four

members of the auxin inducible *PLETHORA* (*PLT*; Aida et al. 2004; Galinha et al. 2007) family have been shown to be essential in determining QC identity in Arabidopsis. Ectopic expressions of *PLT1*, *PLT2* or *SCR* transcription factors or changes in auxin maxima localization result in the formation of an ectopic or displaced QC. These results suggest that QC identity is formed where *SCR* and *PLT* expression overlaps with an auxin maximum (Sabatini et al. 1999; Aida et al. 2004; Blilou et al. 2005).

In addition, the transcription factor WOX5 is critical for stem cell maintenance in the root meristem (Sarkar et al. 2007). *WOX5* is specifically expressed in the four cells of QC, and loss of *WOX5* function in the root meristem stem cell niche causes terminal differentiation in distal stem cells and differentiation of the proximal meristem. It has been thought that a WOX5-dependent signal may move from the QC to the neighbouring stem cells, acting as a homologue of the *WUSCHEL* (*WUS*) gene that maintains stem cells in the shoot meristem in non-autonomous manner. Nevertheless, WOX5 protein has not been localized, probably due to its low abundance, and the possibility of WOX5 acting as a signal cannot be ruled out. Recently, the expression of *WOX5* has been shown to be dependent on the phosphatase *POLTERGEIST* (*POL*) and related *PLL1*, suggesting a requirement of these genes for regulating stem cell maintenance (Song et al. 2008).

5.3.3 Radial Organisation of the Root

The proximal stem cells in the root tip generate the different longitudinal cell files of the root. These files are arranged according to a fixed radial pattern. In the central core of the root, a diarch central vasculature is formed containing two xylem and two phloem poles. These cell files are surrounded by the pericycle, the ground tissues consisting of endodermal and cortical layers and the epidermis, which form root hairs (Fig. 5.4; Dolan et al. 1993). The simple organisation of the root has been elegantly used to create a spatiotemporal transcript map of individual cell types and developmental zones in the root by combining tissue-specific marker lines with cell sorting (Birnbaum et al. 2003; Brady et al. 2007), resulting in a unique tool for biotechnological approaches. Furthermore, several mutants with aberrant radial organisation have been discovered in the past decade, considerably increasing our insight in the tightly controlled process of cell fate determination. Embryos mutated in the *WOODEN LEG* (*WOL*/*CRE1*/*AHK4*) gene contain a reduced number of cells in the vasculature due to aberrant divisions in the vascular primordium (Scheres et al. 1995; Mähönen et al. 2000). This leads to the differentiation of all cell files into protoxylem, creating a symmetrical vasculature compared to the normal diarch symmetry (Mähönen et al. 2000). In addition to their role in QC specification, the GRAS-type transcription factors, SHR (Helariutta et al. 2000) and SCR (Di Laurenzio et al. 1996), are also known for their role in the radial organisation of the root by specifying endodermal and cortical cell fate. SHR is expressed in vasculature, and the protein moves into the neighbouring endodermis and QC

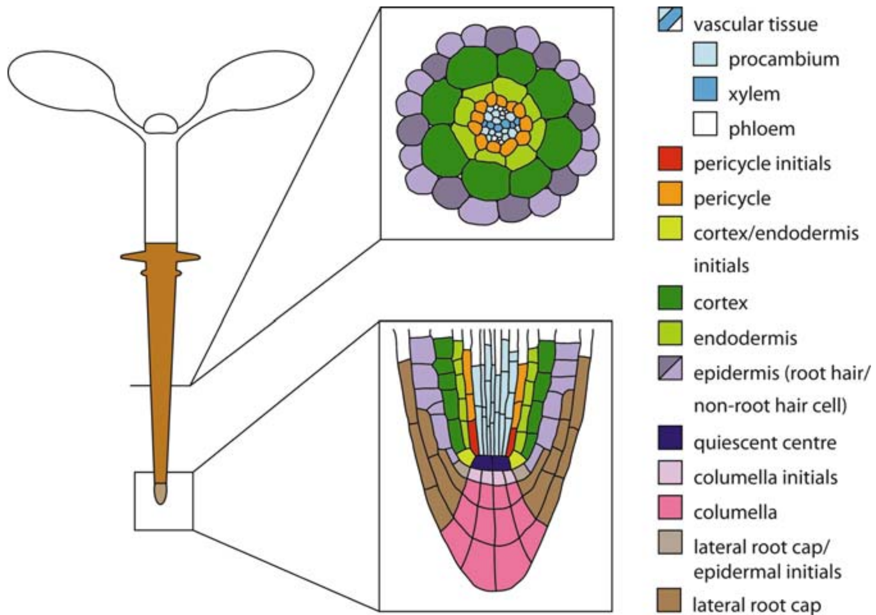

Fig. 5.4 Root meristem and radial anatomy in Arabidopsis thaliana

where it activates the expression of SCR (Nakajima et al. 2001). SCR expression in the endodermis blocks SHR movement into the cortex by sequestering it into the nucleus through protein-protein interaction (Cui et al. 2007). A further study was conducted using microarray analysis on sorted cells from an inducible SHR-GFP line in a *shr-2* background, with subsequent confirmation by ChIP-qPCR. Results showed that the domain of action of SHR and SCR was controlled by the plant-specific zinc finger proteins JACKDAW and MAGPIE, resulting in a complex regulatory network controlling endodermal and cortical cell fate (Levesque et al. 2006; Welch et al. 2007).

5.4 Lateral Root Development

Root branching is one of the most important endogenous determinants of root architecture, as lateral roots make up the greatest part of the root system in many plant species. Although lateral roots have been shown to be initiated from the pericycle in most species, in ferns and maize the endodermis is also involved (Clowes 1961; Bell and McCully 1970). In *A. thaliana* and many other species, lateral roots are initiated from pericycle cells at the xylem pole (Casimiro et al. 2003), whereas in *Daucus carota* and maize they are initiated from phloem pole

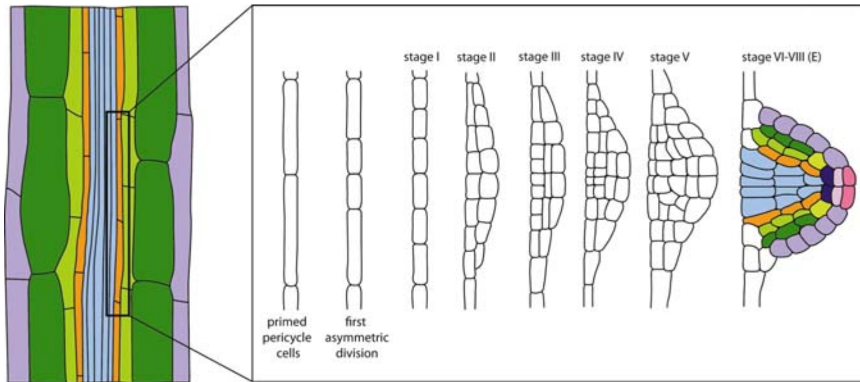

Fig. 5.5 Lateral root development in Arabidopsis thaliana

pericycle cells (Lloret et al. 1989; Casero et al. 1995), although in maize there has been some controversy as to whether lateral roots are initiated from xylem or phloem pole pericycle cells (Bell and McCully 1970; Casero et al. 1995).

The multi-step process of lateral root development begins when two adjacent pericycle cells, referred to as pericycle founder cells (PFCs) within the same longitudinal cell file, undergo almost simultaneous asymmetric anticlinal divisions (Fig. 5.5; Casimiro et al. 2003). As the division occurs in close proximity to the cross wall connecting both PFCs, two small inner cells are generated, flanked by two larger outer cells. The smaller daughter cells form the centre of the future primordium. The outer larger cells undergo an additional asymmetric division, resulting in the formation of a central file of four small cells surrounded by two longer flanking cells (Casimiro et al. 2001). This stage of primordium development is referred to as stage I (Malamy and Benfey 1997), which represents the first visible indication of lateral root-associated patterned cell division in the pericycle. In Arabidopsis, a minimum of three files of the pericycle are usually involved in the early formative asymmetric cell divisions (Casimiro et al. 2003). Subsequent periclinal divisions create an inner and an outer layer (stage II). Further anticlinal and periclinal divisions build a dome-shaped primordium (stages III–VII) that eventually emerges from the parental root (Fig. 5.5, stage VIII; Malamy and Benfey 1997). Although research has been focused mostly on lateral root initiation, recent developments have shed light on both pre- and post-initiation events, allowing the study of lateral root development from a holistic developmental point of view. It has been reported that lateral root emergence is an active process that starts from stage I primordia onwards. Through a cell-type-specific auxin signalling pathway, cell wall remodelling genes are activated to facilitate the emergence of lateral roots through the endodermis, cortex and epidermis (Fig. 5.5; Swarup et al. 2008).

As lateral roots are initiated in the differentiated part of the root, pericycle cells were thought to dedifferentiate before becoming reprogrammed for lateral root formation (Laskowski et al. 1995; Malamy and Benfey 1997). However, although

extensively elongated, PFCs still display typical meristematic features, having three or more vacuoles and a dense cytoplasm with numerous electron-dense ribosomes (Parizot et al. 2008). Furthermore, cell cycle studies have shown that PFCs maintain their mitotic competence in the differentiation zone of the root (DiDonato et al. 2004). In contrast to other pericycle cells that leave the root apical meristem in the G1 phase, the xylem pericycle cells progress to the *G2* phase, suggesting re-entry into the cell cycle at the G2-M transition (Beeckman et al. 2001). However, evidence from recent studies is not in line with this concept and suggests a meristematic state of the pericycle (Dubrovsky et al. 2000; Beeckman et al. 2001; Casimiro et al. 2003). De Smet et al. (2007) discovered the existence of a recurrent auxin maximum, as visualised by the auxin response marker DR5::GUS in the protoxylem cells of the basal meristem to match with the lateral root initiation pattern along the primary root. It has been suggested that this cyclic auxin maximum primes specific pericycle cells to become PFCs and that this signal correlates with the gravity-induced and AUX1-dependent waving of the primary root (De Smet et al. 2007). This hypothesis has been strengthened by mathematical modelling, further suggesting that lateral root initiation and gravistimulation consume the same pool of auxin (Lucas et al. 2008). Arabidopsis mutants with disturbed auxin homeostasis, like *superroot1* (*sur1*; Boerjan et al. 1995), *rooty* (*rty*; King et al. 1995) and *affected lateral root formation1* (*alf1*; Celenza et al. 1995), show strongly aberrant phenotypes at the level of root architecture. This is also the case for mutants disrupted in auxin transport, like *gnom* (Geldner et al. 2004), *aux1* (Bennett et al. 1996; Marchant et al. 1999) and *pin1pin3* (Benkova et al. 2003; Blilou et al. 2005). Furthermore, high concentrations of auxin applied exogenously to the plant disturb the endogenous balanced auxin distribution patterns dramatically and incite pericycle cell division, resulting in excessive lateral root formation (Laskowski et al. 1995; Casimiro et al. 2001).

In summary, these results have led to the notion that a precise auxin distribution pattern responsible for the establishment of gradients is crucial for shaping the final root architecture by guiding the initiation, formation and outgrowth of lateral roots. At the beginning of the lateral formation process, an early auxin maximum has been established in PFCs before the first division events (Benkova et al. 2003). The appearance of this auxin maximum is followed immediately by anticlinal asymmetric divisions of two adjacent PFCs. It is assumed that the newly formed auxin gradient drives a breaking of the PFC symmetry through cell-polarisation events. It has been proposed that the asymmetric division in PFCs is preceded by polar localization of cell nuclei moving towards each other to the site of future cytokinesis (Casero et al. 1993). These early nuclear movements were first described by Kawata and Shibayama (1965) in their pioneering work of lateral root primordium development in rice roots. Results of our preliminary study also show coordinated nuclear migration in two neighbouring pericycle cells prior to the first division of the founder cells in Arabidopsis (Vassileva et al., unpublished data).

The auxin maximum observed in the PFCs is maintained during all later stages of primordium development and it is essential for a successful patterning and outgrowth of the new root. To ensure the correct cellular response, it is necessary

to translate the auxin gradients into a downstream signalling cascade. By binding of auxin to TIR1 (transport inhibitor 1), degradation of small transcriptional repressor proteins of Aux/IAA is promoted (Kepinski and Leyser 2005; Dharmasiri et al. 2005), resulting in the derepression of ARFs and the expression of downstream auxin responsive genes. This may allow auxin to control the expression of several developmental genes directly, including those involved in lateral root initiation (Himanen et al. 2002; Vanneste et al. 2005). Therefore, a missing link in the signalling cascade between auxin perception and response is likely to become problematic for lateral root formation. A classic example is *solitary-root s1r/ iaa14* (*slr1*) that is unable to produce lateral roots. Due to a gain-of-function mutation, this Aux/IAA is not released from its ARF upon auxin stimulus, thereby preventing expression of genes needed for lateral root initiation (Fukaki et al. 2002; Vanneste et al. 2005). Two targets of SLR1/IAA14 have been identified, namely ARF7 and ARF19, and both act as transcriptional activators (Fukaki et al. 2005). Seedlings of the *arf7arf19* double mutant produce only a low number of lateral roots (Wilmoth et al. 2005; Okushima et al. 2007). Recently, *LBD16*/*ASL18* and *LBD29*/*ASL16* (two highly related Lateral organ Boundaries-Domain/Asymmetric Leaves2-like genes) have been reported to be the direct targets of ARF7 and ARF19, and are involved in lateral root initiation (Okushima et al. 2007). The LBD/ASL gene family was originally identified in Arabidopsis, where the family might contain as many as 42 members (Iwakawa et al. 2002; Shuai et al. 2002). Similar genes have also been identified in rice and maize. In rice, *CRL1* has been shown to be highly homologous to *LBD16* and *LBD29* (Inukai et al. 2005). The *crl1* (*crown rootless1*) mutant was isolated in an EMS screen because it did not produce crown roots and formed a reduced number of lateral roots (Inukai et al. 2001; Inukai et al. 2005). In addition to *crl*, another rice mutant, *arl1* (*adventitious rootless1*), deficient in adventitious root formation but with normal lateral root formation, was identified and it appeared to be allelic to *CRL1* (Liu et al. 2005). A maize mutant, *rtcs* (*rootless concerning crown and seminal roots*), has been characterised by the deficiency in embryonic seminal and post-embryonic crown- and brace-root formation (Hetz et al. 1996). It has been speculated that the corresponding gene, *RTCS*, may be the orthologue of *ARL1*/*CRL1* (Taramino et al. 2007). The recent identification of similar genes controlling the same aspect of root development in several distantly related species has raised the question of the existence of conserved mechanisms in root development for all plant species, and underscores the importance of Arabidopsis root research with respect to further potential strategies for root improvement in crop plants.

5.5 Conclusions

This discussion has underlined the importance of an extensive and efficient root system for plant growth and development. In view of exponentially accumulating recent data, the effects of different hormones and specific genes have been

discussed for the formation and shaping of a plant root system. Despite the increasing interest in the last decade for root developmental biology, it became clear that root formation is an enormously complex trait. Novel techniques, such as fluorescence-based cell sorting (FACS), confocal microscopy and chemical genetics, will become increasingly important tools to elucidate the complex transcriptional networks involved in root formation in general and lateral root development in particular. Furthermore, a good understanding of root development will provide insight into the mechanisms of how plants deal with their ever-changing and complex environment.

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Chapter 6
Legume Nodule Development

K. D'haeseleer, S. Goormachtig, and M. Holsters

6.1 Introduction

Parallel to the quickly growing global human population, demands for higher crop yields are starting to become an urgent necessity to address. The use of increasing amounts of nitrogen and phosphorus fertilizers will inevitably lead to eutrophication of the environment and the atmospheric accumulation of greenhouse gases such as dinitrogen oxide. Changes in terrestrial and aquatic ecosystems might place a heavy burden on our environmental future. Hence, more sustainable solutions for growing "high-yield" plants on less fertile grounds are essential.

Plants may survive on nutrient-poor soils by cooperating with other organisms such as bacteria, algae or fungi. Arbuscular mycorrhiza (AM) establish a symbiotic interaction between the majority of land plants and fungi of the order Glomales, improving uptake of phosphorus by the plant. Also well studied is the root nodule symbiosis between members of the Fabaceae and Gram-negative bacteria, collectively called rhizobia. Under nitrogen-limited conditions, the rhizobia-legume interaction leads to the formation of new root organs, the nodules, on the host plant. Inside the nodules, nitrogen-fixing rhizobia convert atmospheric nitrogen into compounds usable by the plant. In return, the bacteria are provided with carbon and find a protected environment.

K. D'haeseleer, S. Goormachtig, and M. Holsters
Department of Plant Systems Biology, Flanders Institute for Biotechnology (VIB), and
Department of Plant Biotechnology and Genetics, Ghent University Technologiepark 927, 9052 Gent, Belgium
e-mail: marcelle.holsters@psb.vib-gent.be

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6.2 Evolution Towards Nitrogen-Fixing Bacterial Endosymbiosis

Although root nodule symbiosis (RNS) is best studied in legumes interacting with rhizobia, the process is not restricted to this plant family. *Parasponia* sp., belonging to the Ulmaceae, is the only non-legume that nodulates in the presence of certain rhizobial strains. Moreover, the Gram-positive actinomycetes *Frankia* sp. are able to form nodules on nearly 200 species of so-called actinorhizal plants belonging to eight different families, namely the Betulaceae, Casuarinaceae, Coriariaceae, Datiscaceae, Elaeagnaceae, Myricaceae, Rhamnaceae and Rosaceae (Doyle et al. 1997).

Analysis of phylogenetic trees based on sequences of the ribulose-1,5-bisphosphate carboxylase/oxygenase chloroplast (*rbcL*) gene indicated that all plant families performing RNS belong to a single "nitrogen-fixing" clade within Eurosid I, designated the FaFaCuRo clade because it includes Fabales, Fagales, Cucurbitales and Rosales (Soltis et al. 1995). Cyanobacteria of the genus *Nostoc* fix nitrogen symbiotically in leaf glands of *Gunnera* sp. The Gunneraceae do not belong to the Eurosid I clade and represent a nitrogen-fixing symbiosis that evolved independently from the actinorhiza–*Frankia* and rhizobia-legume symbioses (Soltis et al. 1995; Kistner and Parniske 2002).

The restricted occurrence of nodulators suggests a common ancestor with a "predisposition to nodulate", a genetic background that enabled plants to evolve towards RNS (Soltis et al. 1995). Within the FaFaCuRo clade, nodulating genera are a vast minority, scattered among non-nodulating families and genera. Rhizobia-legume and actinorhiza–*Frankia* symbioses fall into distinct lineages of the nitrogen-fixing clade. Whereas legumes are all members of one subclade, actinorhizal plants are dispersed in three subclades amongst the non-nodulating plant species. This distinctness is reflected in the different structure/ontogeny of leguminous and actinorhizal nodules. For instance, nodules on legume roots originate from divisions in the root cortex and pericycle, and develop into stem-like organs with a peripheral vasculature, whereas actinorhizal nodules are formed by modification of lateral roots and have a central vascular bundle. This different structure, together with the presence of many non-nodulators, suggests that nitrogen-fixing nodulation has originated several times independently (Swensen 1996).

The Fabaceae are traditionally divided in three subfamilies, the monophyletic Mimosoideae and Papilionoideae and the paraphyletic Caesalpinioideae. Most members (90%) of the monophyletic clades are known to nodulate, while only a minority of species of the Caesalpinioideae form nodules in the presence of rhizobia. Based on the occurrence of nodulation and phylogenetic analysis, three single and independent origins of nodulation within the Leguminosae have been proposed (Doyle et al. 1997). The legume nodulation process consists of two interlinked developmental programmes: an epidermal programme, involving infection and entry of bacteria, and a cortical programme comprising formation of the nodule primordium and subsequent development (Guinel and Geil 2002).

The existence of mutants defective in one of the two programmes and the occurrence of spontaneous nodules (with similar structures but completely devoid of bacteria) suggest the independent origin of the two programmes.

The epidermal programme has features in common with AM (Guinel and Geil 2002). During this symbiosis, fungi penetrate the root epidermis and grow towards the inner cortex to form intracellular arbuscules where mutual exchange of nutrients takes place (Reinhardt 2007). AM has evolved more than 350 million years ago, long before the predisposition of nodulation (Kistner and Parniske 2002). As several genes essential for both AM and RNS have been identified, parts of the infection pathway of nodulation might have evolved from the older and more common AM process (Kistner and Parniske 2002). On the other hand, *SYMRK*, a common gene involved in early responses in both endosymbioses, exists in at least three different structural versions. Whereas the shorter versions are sufficient to support AM, the longest version is required for root nodule symbiosis. As the latter SYMRK version is solely present in all tested (non)-nodulating eurosids, this gene might be involved in the proposed genetic predisposition (Markmann et al. 2008).

Infection thread formation, nodule primordium formation and nodule development may have recruited functions from other existing organ formation processes. For example, the process leading to pollen tube growth has a lot in common with infection thread formation in both legumes and actinorhizal plants and might have been hijacked for root nodule symbiosis (Rodriguez-Llorente et al. 2004). Also, actinorhizal nodules arise from modified lateral root structure and, in *Medicago truncatula* (barrel medic), the lateral root organ-defective (*Mtlatd*) mutant provides a genetic link between the nodule meristem and lateral root meristem (Bright et al. 2005).

Important crop legumes, such as soybean (*Glycine max*), bean (*Phaseolus vulgaris*), pea (*Pisum sativum*) and lentil (*Lens culinaris*), are difficult to study because of their large genomes and low transformation capacities. *M. truncatula* and *Lotus japonicus* are the accepted model legumes in which nodule formation is studied. Both plants have a small diploid genome (470–550 Mp) and a short life cycle, are self-fertile and can be transformed. *M. truncatula* belongs to the inverted repeat clade, is closely related to temperate legumes, such as clover (*Trifolium* sp.), pea, vetch (*Vicia* sp.), chickpea (*Cicer arietum*) and lentil, but like *L. japonicus*, is more distantly related to tropical climate legumes, such as soybean and bean (Phaseolid clade; Ané et al. 2008). Because of its economic importance and the phylogenetic proximity to other major crops, soybean is proposed as a third model legume in addition to *M. truncatula* and *L. japonicus*.

6.3 Legume Nodule Initiation and Development

The rhizobia-legume symbiosis is initiated by plant root exudates that support the growth of rhizobia and trigger expression of crucial nodulation genes. Among these exudates are flavonoids (e.g. luteolin, narigenin and genistein) that specifically

interact with bacterial NodD proteins to activate the expression of nodulation (*nod*) genes, leading to the formation and secretion of nodulation (Nod) factors (D'Haeze and Holsters 2002).

Nod factors (NFs), the key signal molecules that provoke nodulation responses in the host plant, are lipochitooligosaccharides with an oligomeric backbone of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) residues and an acyl chain at the non-reducing terminal residue. NFs vary in number of GlcNAc residues, as well in the nature of the acyl group and in substituents at (non)-reducing terminal residues. Almost all nodulating rhizobia have the *nodABC* genes in common. NodC synthesizes the chitooligosaccharide; NodB and NodA deacetylate and N-acylate the backbone structure respectively. Modifications of the NF structure depend on strain-specific nodulation genes encoding enzymes that synthesize precursors used by transferases (e.g. *nodH*, *nodPQ* and *nodL*). The *nodIJ* genes are responsible for secretion of the NFs. The differences in NF structure play an important role in the host specificity of rhizobial strains (D'Haeze and Holsters 2002; Geurts and Bisseling 2002).

Purified NFs provoke several responses in susceptible root hairs. Within seconds to minutes after NF application, root hair cells respond with a Ca^{2+} influx at the tip, increasing the cytosolic Ca^{2+}, immediately followed by Cl^{-} and K^{+} effluxes. These ion movements correlate with plasma membrane depolarization and extracellular alkalinization (Felle et al. 1999). Calcium is known to influence polarized root tip growth and, thus, may be involved in redirection of the root hairs during symbiosis (Esseling et al. 2003). Ten to 15 min after NF application, another calcium response, Ca^{2+} spiking, occurs independently from the Ca^{2+} influx. This process comprises rhythmic oscillations of Ca^{2+} in and around the nucleus, and is needed for the activation of downstream responses (Miwa et al. 2006a; Sun et al. 2007).

The earliest morphological event, perceived 1 h after NF application, is the growth arrest of the root hair tip associated with root hair swelling and deformation. NF-dependent reorganization of the actin cytoskeleton and microtubule network may redirect the vesicle traffic away from the centre of the apical dome of the root hair, thereby changing its growth direction (Gage 2004). The so-called root hair curling (RHC) infection mode starts when rhizobia attach to developing root hairs in the susceptible zone I of the root, and trigger root hair deformation and curling to form a closed compartment harbouring a bacterial microcolony. Within the curl, local degradation of the plant cell wall and invagination of the plasma membrane lead to the formation of an infection thread (IT), a tubular structure through which dividing rhizobia embedded in the plant's extracellular matrix are guided towards the basal side of the epidermal cell. There, the IT fuses with the distal cellular membrane, releasing the bacteria into the intercellular space between the epidermis and outer cortex cell layers. Via invagination and tip growth of the underlying cells, similarly to the process in the epidermis, the IT branches and proceeds towards the cortex of the root where a nodule primordium is formed (Gage 2004).

The process of IT formation has been analyzed to some extent at the cellular level. In pea, vetch, alfalfa (*Medicago sativa*) and *L. japonicus*, cells in the outer

cortex re-enter the cell cycle but arrest in the G2 phase (Timmers et al. 1999). The nucleus moves from the periphery to the centre of the cell surrounded by the cytoplasm, creating columns with radially aligned cytoplasmic bridges or pre-infection threads, which are followed by the ITs migrating through the outer cortex to the inner root where an incipient nodule primordium is created (van Brussel et al. 1992; Timmers et al. 1999; van Spronsen et al. 2001). Parallel to RHC and IT formation, bacterial NFs reactivate the cell cycle in the inner cortex adjacent to the xylem poles where, via anticlinal and periclinal divisions, a nodule primordium is formed. Reactivation of pericycle and endodermal cells, adjacent to the primordium, leads to vascularization of the newly developing organ (Brewin 2004).

Upon reaching cells of the nodule primordium, bacteria are released from the ITs through unwalled outgrowths of the IT beneath a rupture point or at the IT tip, called infection droplets and infection pegs respectively (Brewin 2004). During their internalization, rhizobia are enveloped by a peribacteroid or symbiosome membrane that is originally derived from the plant plasma membrane, but becomes enriched with specific proteins and lipids to form an interphase for metabolite exchange between differentiated N_2-fixing bacteria, called bacteroids, and the plant host cells (Catalano et al. 2004, 2007). Depending on the host plant, symbiosomes divide and/or bacteroids divide within the symbiosome, resulting in symbiosomes with only one or many bacteroid(s) respectively. After a while, the infected cells are filled with symbiosomes in which bacteroids are responsible for nitrogen fixation (Brewin 2004).

Although many legumes are nodulated through the mechanisms described above, variations occur in the legume family. For instance, two types of nodules exist, indeterminate and determinate ones. Some tropical and subtropical legumes, such as soybean, bean and *L. japonicus*, form determinate nodules. This nodule type lacks a persistent meristem and grows mainly through cell expansion, rather than cell division. Moreover, determinate nodules develop from the outer cortex (Crespi and Gálvez 2000). Examples of legumes that form indeterminate nodules are *M. truncatula*, alfalfa, pea and other, mostly temperate legumes. In contrast to determinate nodules, indeterminate nodules have a persistent meristem that continuously provides new cells to the nodule. Hence, these nodules consist of several developmental zones. Distal from the meristem, an infection zone is observed where ITs grow and release bacteria. This zone is followed by a fixation zone with infected and uninfected cells and where nitrogen fixation takes place. In the proximal senescence zone, symbiosomes and host cells are degraded for nutrient recycling (Van de Velde et al. 2006). The central zones are surrounded by nodule parenchyma that contains vascular tissues, via which nutrients are exchanged between symbionts and the host plant.

Also variation in infection mode is observed between legumes and within one legume, depending on the physiological growth conditions, as nicely demonstrated by the analysis of the symbiosis of the tropical legume *Sesbania rostrata*. This plant develops indeterminate nodules via RHC infection when grown under well-aerated conditions, but changes mode of infection under waterlogged conditions, when the RHC infection mode and the nodule meristem activity are inhibited by ethylene.

Hence, under these conditions, the plant provides an alternative invasion/nodulation mechanism, using of crack-entry at lateral root bases (LRBs). Rhizobia intrude the root via cracks formed by protrusion of lateral or adventitious roots, and colonize large intercellular spaces called infection pockets. Subsequently, inter- and intracellular ITs grow towards the nodule primordium. A very similar process occurs in *Neptunia* sp., whereas direct uptake from the infection pockets by nodule primordium cells occurs in jointvetch (*Aeschynomene* sp.) and *Arachis* sp. (Goormachtig et al. 2004a).

6.4 NF Perception, Signal Transduction and Genes Involved in the Establishment of Nodulation

The main players in NF perception and signalling have been elucidated. The first cellular and genetic experiments, using bacterial and plant mutants with defects in NF production and perception respectively, had suggested that different NF perception complexes might be involved because distinct responses required diverse NF concentrations or structural features. For instance, Ca^{2+} influx was activated by 1 nM of NFs whereas Ca^{2+} spiking was induced at concentrations of 1 to 10 pM of NFs (Shaw and Long 2003). The *Sinorhizobium meliloti nodL nodF* and *Rhizobium leguminosarum nodO nodE* double mutants generating NFs with a C-18:1 N-acyl group, rather than C-16:2, induced all early responses (root hair deformation, RHC, early nodulation (ENOD) induction, cortical cell division), but failed to form ITs (Ardourel et al. 1994; Walker and Downie 2000). Moreover, in pea, the *SYM2A* allele was needed to perceive acetylated NFs, and played a role in IT growth but not in early responses (Walker et al. 2000). Ardourel et al. (1994) proposed the existence of a low-affinity, non-stringent "early" receptor, involved in induction of epidermal signalling responses, and a high-affinity/ stringent "entry" receptor controlling invasion of bacteria with high structural NF demands.

6.4.1 The Search for NF Receptors

Based on biochemical studies, two NF-binding site proteins, NFBS1 and NFBS2, were detected in root extracts of *M. truncatula* and cell suspension cultures of *M. varia* respectively (Bono et al. 1995; Niebel et al. 1997). NF receptors were identified via the analysis of mutants that lack all early nodulation responses, such as *SYM10* of pea, NF receptor 1 (*NFR1*) and *NFR5* of *L. japonicus*, and Nod Factor Perception (*NFP*) of *M. truncatula* (Walker et al. 2000; Amor et al. 2003; Radutoiu et al. 2003).

In *L. japonicus*, neither the *nfr1* nor the *nfr5* mutants formed ITs and nodule primordia. Both mutants had no early cellular responses, such as root hair deformation and RHC, upon addition of *M. loti* strains or purified NFs. In *Ljnfr5* mutants, depolarization of root hairs and extracellular alkalinization were not detectable, whereas they were attenuated in *Ljnfr1* mutants. These observations suggested that the *NFR1* and *NFR5* genes were equally necessary in NF perception (Madsen et al. 2003; Radutoiu et al. 2003).

LjNFR5, orthologous to *PsSYM10* and *MtNFP*, encodes a LysM-receptor-like kinase (RLK) with three extracellular LysM motifs. LysM domains have been shown to bind the *N*-acetylglucosamine backbone of peptidoglycan and chitin, making the LysM-RLKs good candidates as NF receptors (Bateman and Bycroft 2000), but direct binding of NFs has not yet been demonstrated. LjNFR5 is an atypical member of the LysM-RLKs because it lacks an activation loop that usually regulates kinase activity and has, hence, been proposed to interact with another RLK to induce downstream signalling cascades via phosphorylation (Madsen et al. 2003).

In contrast, *LjNFR1* encodes a LysM-RLK with two conserved and one more variable extracellular LysM motif, and a serine/threonine kinase anchored via a transmembrane segment (Radutoiu et al. 2003). Expression of *LjNFR1* and *LjNFR5* in *M. truncatula* and *L. filicaulis* allowed nodule organogenesis upon inoculation of *M. loti*, the microsymbiont of *L. japonicus*. Moreover, the LysM domains appeared to be crucial for specific recognition of the rhizobial signal, and the LysM2 of LjNFR5 was able to discriminate between different NFs (Radutoiu et al. 2007). Based on these results, the mutant phenotypes and the predicted protein structures, LjNFR1 and LjNFR5 are believed to constitute a heteromeric NF receptor complex (Radutoiu et al. 2003, 2007). Binding of NFs to the extracellular LysM domains and the subsequent activation of the kinase domain of NFR1 will trigger a downstream signalling cascade that activates nodulation (Parniske and Downie 2003; Madsen et al. 2003; Radutoiu et al. 2003).

In *M. truncatula* and pea, the situation might be slightly different because only the genes *MtNFP* and *PsSYM10* respectively, orthologues of the signalling NF receptor *LjNFR5*, were identified as being essential for all early NF-induced responses (Amor et al. 2003; Madsen et al. 2003). In accordance with the phenotype of *Ljnfr5*, the *Mtnfp* mutant had lost all early nodulation responses (Ben Amor et al. 2003; Arrighi et al. 2006). The presence of transcripts in the infection zone of indeterminate nodules suggested that MtNFP might also be involved later during nodulation at the level of IT growth (Arrighi et al. 2006).

A *sym2* mutant of pea showed RHC and entrapment of rhizobia, but no IT formation, making PsSYM2 a good candidate as entry receptor. The orthologous *SYM2* region in *M. truncatula* was mapped by Limpens et al. (2003) and contained seven genes coding for the LysM domain containing RLKs (LYK1 to LYK7). Via RNA interference (RNAi), only two genes, *LYK3* and *LYK4*, were shown to be involved in nodulation (Limpens et al. 2003). LYK3 and LYK4 showed high homology to LjNFR1 and, hence, are candidates to interact with MtNFP for early NF responses. On the other hand, LYK3 and LYK4 might also be involved later in

nodulation as entry receptor, because silencing of these genes caused aberrant IT growth and morphology, whereas early NF responses were normal (Limpens et al. 2003). Smit et al. (2007) demonstrated that the Hair Curling Locus (HCL) of *M. truncatula* encodes MtLYK3. Three allelic mutants had similar RHC-deficient phenotypes, whereas the weaker mutant allele *hcl4* had microcolonies in normal root hair curls, but impaired IT growth (Smit et al. 2007). In none of the *hcl* mutants were the early nodulation responses altered, and experiments with the *S. meliloti nodF nodE* and *nodL* mutants revealed that the HCL function depends on NF structure and concentration, supporting the hypothesis of MtLYK3 as an entry receptor.

MtNFP and MtLYK3 are also believed to be involved at later steps of the infection process. Reducing the *MtNFP* transcript level resulted often in aborted ITs in the root hair and in ITs with aberrant "sac"-like structures. *hcl4* mutants, bearing a mutant *MtLYK3*, also showed this distorted IT growth, implying a role for both genes in IT growth and/or bacterial release (Arrighi et al. 2006; Smit et al. 2007).

Besides *MtNFP* and the seven *LYK* genes, the genome of *M. truncatula* contains nine other genes encoding LysM-RLKs, of which six were expressed predominantly in roots and nodules (Arrighi et al. 2006). In *Arabidopsis thaliana*, a LysM-RLK is involved in resistance to both fungal and bacterial pathogens through chitin signalling (Miya et al. 2007; Wan et al. 2008). Based on these observations and phylogenetic analysis, NF recognition and chitin perception might be evolutionarily related, and similar perception mechanisms might be used in symbiosis and immune responses (Zhang et al. 2007; Wan et al. 2008).

6.4.2 NF Signalling

6.4.2.1 Ca^{2+} Spiking

Upon perception of NFs, a rhythmic oscillation of the Ca^{2+} concentration, a process called Ca^{2+} spiking, is observed in and around the nucleus of a root hair cell (Oldroyd and Downie 2008). A single Ca^{2+} spike consists of a rapid increase, presumably caused by opening of a Ca^{2+} channel from an internal store, followed by a slow decrease in Ca^{2+} concentration, pointing at a slow re-uptake of the cytosolic Ca^{2+} by the store (Ehrhardt et al. 1996). In animal systems and also in plant stomatal cells, the amplitude of the spikes and the period between them encode information that can be translated into activation of downstream responses (Peiter et al. 2007). Also during nodulation, Ca^{2+} spiking is needed to activate downstream responses. Pharmacological antagonists that interfere with intracellular ion channels and Ca^{2+} spiking, such as 2-aminoethyl diphenylborate (2-APB), cyclopiazonic acid (CPA) and 8-(N,N-diethylamino) octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8), block Ca^{2+} spiking and also expression of *ENOD* genes, such as *ENOD11*. These observations pinpoint Ca^{2+} spiking within the NF

signalling pathway and upstream of *ENOD* gene activation (Charron et al. 2004). Moreover, the period between Ca^{2+} oscillations has been shown to determine the nature of downstream responses, and 36 sequential Ca^{2+} spikes appear to be required for nodulation gene expression (Miwa et al. 2006b; Sun et al. 2007). In *S. rostrata*, in which cracks at LRBs can be invaded by rhizobia, faster and more symmetrical Ca^{2+} oscillations were observed in epidermal cells at LRBs than during root hair invasion. Diminishing Ca^{2+} spiking frequency by enhanced jasmonic acid (JA) or reduced ethylene levels stimulated intracellular root hair invasion, but prevented nodule formation. Hence, intracellular invasion in root hairs seems to be linked with a very characteristic Ca^{2+} signature (Capoen et al. 2009).

6.4.2.2 Phospholipid Signalling

Pharmacological experiments provided evidence for the participation of heterotrimeric G proteins in NF signalling because agonists of G proteins, such as mastoparan and Mas7, were able to mimic NF-induced *ENOD11* and *ENOD12* induction in *M. truncatula*, whereas an antagonist, such as the pertussis toxin, blocked both NF and mastoparan activity (Pingret et al. 1998). However, these results have to be interpreted with care because, more recently, mastoparan effects in plants have been shown to occur independently of G proteins via MAP kinase signalling (Miles et al. 2004). Alternatively, phospholipase C (PLC), which is activated by G proteins and cleaves the membrane phosphatidylinositol (4,5)-bisphosphate (PIP_2) into the possible secondary messengers inositol (1,4,5)-triphosphate (IP_3) and diacylglycerol (DAG), might play a role in transduction of responses to NFs, because inhibitors block the NF activity. Thus, G-protein-mediated activation of PLC can cause Ca^{2+} changes in the cytosol by mobilization of IP_3 (Pingret et al. 1998). In vetch, application of NF or mastoparan increased the concentration of phosphatidic acid (PA) and DAG, pointing at activation of phospholipase D (PLD) and PLC respectively (den Hartog et al. 2001). In *M. truncatula*, *ENOD11* expression is reduced by application of inhibitors of PLC and PLD, underlining a central role for multiple phospholipid signalling pathways in NF signal transduction (Charron et al. 2004).

6.4.2.3 Common SYM Pathway

In *M. truncatula*, three genes, Does not Make Infections 1 (*DMI1*), *DMI2* and *DMI3* have been shown to be involved in early signalling stages of nodulation. Analysis of early NF responses within the *Mtdmi1*, *Mtdmi2* and *Mtdmi3* mutants, and isolation of the corresponding genes gave insight into the progression of NF signalling after perception. NF application still provoked root hair deformation in all three mutants, placing the *DMI1*, *DMI2* and *DMI3* downstream of the NF perception (Oldroyd and Downie 2004). Moreover, *MtDMI3* was placed downstream of *MtDMI1*, *MtDMI2*

and Ca^{2+} spiking, because the latter response was still observed in *Mtdmi3* but not in *Mtdmi1* and *Mtdmi2* mutants (Lévy et al. 2004; Mitra et al. 2004b). Based on these analyses, *MtDMI1* and *MtDMI2* might be essential for the induction of Ca^{2+} spiking.

Cloning of *MtDMI1* and its homologues *CASTOR* and *POLLUX* in *L. japonicus* revealed homology to the prokaryotic *Methanobacterium thermoautotrophicum* potassium channel (MthK) Ca^{2+} gated K^{+} channel (Ané et al. 2004; Imaizumi-Anraku et al. 2005). Analyses of *dmi1* mutants and MtDMI1:green fluorescent protein (GFP) fusions suggest a possible role of DMI1 in regulating Ca^{2+} channel activity following NF perception (Riely et al. 2007; Oldroyd and Downie 2008). CASTOR and POLLUX were shown to form two independent homocomplexes and CASTOR to be localized at the nuclear envelope in *Lotus* cells (Charpentier et al. 2008). Also *MtDMI1:eGFP* fusions suggested a perinuclear localization (Riely et al. 2007). Moreover, recently both *CASTOR* and *POLLUX* genes were suggested to encode potassium-permeable channels (Charpentier et al. 2008), possibly triggering the opening of calcium release channels or functioning as counter-ion channels to compensate for the charge release during the calcium efflux.

MtDMI2 and its orthologues *PsSYM19*, *LjSYMRK* (symbiosis RLK), *SrSYMRK* and *MsNORK* (nodulation receptor kinase) encode an RLK with an amino-terminal signal peptide, a transmembrane domain, an intracellular serine/threonine kinase domain, and an extracellular leucine-rich repeat domain (Endre et al. 2002; Stracke et al. 2002; Capoen et al. 2005). These leucine-rich motifs are believed to play a role in specific protein-protein interactions. *Dmi2* mutants reacted normally to NFs, but RHC stopped when the root hair tip touched its own shank blocking bacterial entrapment (Esseling et al. 2004). Besides a function in the early NF signalling cascade, MtDMI2 also influences symbiosome formation. In well-formed nodules, *MtDMI2* was expressed in both the host cell membrane and the IT membrane in the nodule apex, adjacent to the meristem where bacterial release and symbiosome formation occur. Also partial knock-down of MtDMI2 via RNAi resulted in formation of ITs, but blocked symbiosome formation (Limpens et al. 2005). Similar results were observed in *S. rostrata*.

The third *M. truncatula* gene, *DMI3*, placed downstream of Ca^{2+} spiking, encodes a calcium calmodulin-dependent protein kinase (CCaMK) that has three EF-hand-binding domains, a calmodulin (CaM)-binding domain for direct and indirect Ca^{2+} binding and a large kinase domain for auto- and substrate phosphorylation (Lévy et al. 2004; Mitra et al. 2004b). Binding of free Ca^{2+} ions induces autophosphorylation, which subsequently enhances CaM binding, followed by substrate phosphorylation. An autoinhibition domain overlaps the CaM-binding domain and negatively regulates DMI3 activity, whereas removal of this domain causes constitutive DMI3 activity (Gleason et al. 2006). Also, deletion of the Ca^{2+}- and CaM-binding sites, as well as point mutations in the autophosphorylation domain result in the constitutive activation of nodulation genes and the formation of spontaneous nodules in the absence of rhizobia. Thus, the protein activation through Ca^{2+} is necessary and sufficient for the induction of nodule morphogenesis (Gleason et al. 2006; Tirichine et al. 2006). Although *DMI3* alleles harbouring only

the kinase domain are able to induce nodules, they do not allow bacterial entry, indicating essential and subtle roles for CaM- and Ca^{2+}-binding domains during rhizobial infection (Gleason et al. 2006).

MtDMI3 is expressed in roots, and enhanced in the cell layers adjacent to the nodule meristem (Lévy et al. 2004; Limpens et al. 2005). Subcellularly, it is located in the nucleus of root hair cells where Ca^{2+} spiking occurs, which fits its putative role of decoding Ca^{2+} spiking to cause downstream responses (Oldroyd and Downie 2006). Recently, a nucleus-localized protein, Interacting Protein of DMI3 (IPD3/CYCLOPS), has been shown to interact with DMI3, possibly via a C-terminal coiled-coil domain, and to form a complex together with GRAS proteins on DNA (Messinese et al. 2007; Oldroyd and Downie 2008). In *cyclops* mutants, upon application of rhizobia, microbial infections were inhibited and nodule organogenesis was only transiently initiated. Moreover, spontaneous nodulation was completed upon introduction of a mutated *CCaMK*, suggesting that *cyclops* mutants block infection but are still able to develop nodules or, in other words, that a bifurcation occurs downstream of CCaMK (Charpentier et al. 2008).

Besides the genes described above, two more genes, nucleoporin 133 (*Nup133*) and *Nup85*, have been identified in *L. japonicus* as early nodulation signalling components. The former encodes a nucleoporin that is localized in the nuclear envelope of root hair cells; its mutation results in deficient nodulation (cf. reduced frequency and efficiency) in a temperature-dependent way. *Nup133* is placed upstream of Ca^{2+} spiking because it is needed for Ca^{2+} spiking (Kanamori et al. 2006). *nup85* mutants also fail to perform Ca^{2+} spiking and form few or no nodules. NUP85 and NUP133 are believed to function together in the same subcomplex, allowing macromolar transport across the nuclear envelope (Saito et al. 2007).

Altogether, common *SYM* genes take part in the translation of NF and unknown fungal signal into Ca^{2+} spiking, which is perceived by CCaMK/DMI3 that transduces the signals towards downstream genes regulating nodulation and AM respectively. Differential Ca^{2+} responses might control the outcome of the signalling cascade because the variability in spike duration was shown to provide flexibility to activate different processes (Kosuta et al. 2008).

6.4.3 Transmitting the Signal

Although recently the central players in perception and early transduction of the nodulation signals have been elucidated, little is known about the processes that link NF perception with nodule formation. Approximately 7% of the coding sequence of the plant genomes encodes predicted transcription factors (TFs), illustrating that a non-negligible part of gene regulation is executed at the transcriptional level. Many developmental processes and plant responses are regulated via TFs. Hence, studying TFs that are involved at early stages of nodulation might identify central molecules that link NF perception to gene expression.

6.4.3.1 NSP1 and NSP2

The TFs Nodulation Signalling Pathway 1 (NSP1) and NSP2 are involved at early stages of the nodulation programme. Mutation in the genes of both *L. japonicus* and *M. truncatula* cause a Nod^{-}/Myc^{+} phenotype. Whereas (reduced) root hair deformations and Ca^{2+} spiking are observed, these mutants lack rhizobial infection and cortical cell division, and show reduced or blocked nodulin gene expression (Catoira et al. 2000; Oldroyd and Long 2003; Heckmann et al. 2006). Moreover, the mutations interfere with spontaneous nodule formation induced by overexpression of the CCaMK gain-of-function construct, indicating that NSP1 and NSP2 act downstream of the common SYM pathway (Gleason et al. 2006).

NSP1 and *NSP2* encode GRAS-like TFs, a plant-specific family generally implicated in signal transduction, meristem maintenance and developmental processes. NSP proteins contain a conserved C-terminal region possibly involved in protein-protein and protein-DNA interactions (Bolle 2004). The N-terminal region of the GRAS proteins is believed to function as an activation domain and is highly variable, reflecting the different requirements for interactions with different signalling proteins in various developmental pathways (Pysh et al. 1999).

MtNSP1 is located on chromosome 8, and shows high homology to SCARECROW-LIKE 29 of *Arabidopsis* and other genes in rice (*Oryza sativa*) (homologue of NSP1, *OsHNO*) and poplar (*Populus trichocarpa*) (*PtHNO1*, *PtHNO2*). The occurrence of homologues in non-legume plants implies that the gene is recruited from a non-symbiotic pathway during evolution (Smit et al. 2005). *NSP1* is preferentially expressed in roots, and its expression in *M. truncatula* does not markedly change upon addition of rhizobia until 2 days post-inoculation (dpi); in *L. japonicus*, the transcript level of *LjNSP1* increases from 4 dpi onwards. Still, in both cases the gene was continuously expressed throughout the later stages of nodulation, suggesting that NSP1 plays a role that goes beyond early signalling, and that the TFs might be required for maintenance of infection and/or nodule development (Heckmann et al. 2006). MtNSP1:GFP fusion revealed localization in the nucleus, which is consistent with a possible role as TF-binding DNA and a putative target of MtDMI3 (Smit et al. 2005; Oldroyd and Downie 2008). Indeed, NSP1 was shown to bind the *ENOD11* promoter as well as the promoters of ERF Required for Nodulation 1 (ERN1) and Nodulation Inception (NIN), two other TFs acting downstream of CCaMK/DMI3 (Hirsch et al. 2009).

MtNSP2 was mapped on chromosome 3, and shows only 17% identity and 32% similarity to *MtNSP1* (Kaló et al. 2005). *MtNSP2* is expressed in shoots as well as in roots, and is induced after rhizobial inoculation. The orthologue *LjNSP2* is transiently and specifically expressed from 2 dpi onwards, suggesting that it is specialized in nodule initiation (Kaló et al. 2005; Murakami et al. 2006). A MtNSP2:GFP fusion protein localizes to both the nuclear envelope and the endoplasmic reticulum, and its location shifted towards the nucleus upon addition of NFs or rhizobia (Kaló et al. 2005). MtNSP2 associated (but without direct DNA binding) with the *ENOD11* promoter and MtNSP1 and MtNSP2 formed homodimers as well as heterodimers. Also MtNSP2 was required for NSP1-ENOD11

promoter interaction, suggesting that NSP1 and NSP2 form a complex to bind the ENOD11 promoter, thus explaining their similar but non-redundant function in early nodulation signalling (Oldroyd and Downie 2008; Hirsch et al. 2009). As MtNSP1 and MtNSP2 binding to the ENOD11 promoter is enhanced by NF treatment and both genes are required for CCaMK/DMI3-induced gene expression, both proteins are probably activated by CCaMK/DMI3, possibly via phosphorylation (Kaló et al. 2005; Smit et al. 2005; Heckmann et al. 2006; Oldroyd and Downie 2008; Hirsch et al. 2009).

6.4.3.2 NIN

Characterization of a transposon-tagged mutant in *L. japonicus* revealed another predicted TF, Nodule Inception (NIN), with an important role in nodulation. In the NF signalling pathway, NIN was positioned downstream of Ca^{2+} spiking and required all early signalling components of the NF signalling pathway (Schauser et al. 1999; Marsh et al. 2007).

Ljnin mutants showed excessive root hair deformation and curling in an enlarged sensitive root zone, but never showed IT formation or cortical cell division. Orthologues of *LjNIN* were identified in pea (Borisov et al. 2003) and *M. truncatula* (Marsh et al. 2007), and the corresponding mutants revealed phenotypes comparable to those of *Ljnin*. Expression analysis showed increasing transcript levels from 30 min after application of NFs or rhizobia onwards, and a high expression level at several dpi. In nodules of *L. japonicus*, *NIN* transcripts are located in nodule primordium cells, in the nodule parenchyma and vascular bundles, and in infected and non-infected cells of mature determinate nodules. Expression of *PsNIN* (*PsSym35*) was detected in the meristem and infection zone (Borisov et al. 2003). These data suggest that NIN might also be needed later during nodule development. Spontaneous nodule development elicited by ectopic expression of a gain-of-function CCaMK construct is abolished in a *nin* mutant background, implying that NIN is required for nodule formation and might act as a positive regulator of both bacterial entry and primordium development (Marsh et al. 2007).

At the same time, NIN might be a negative regulator of the early NF responses (Marsh et al. 2007). In *Mtnin1-1* mutants, Ca^{2+} spiking responses and expression of the promoter *ENOD11*:β-glucuronidase (*uidA*) fusion at 12 h post-inoculation (hpi) were comparable to those of wild-type plants. However, at later time points, *uidA* expression extended outside the normal response zone, correlating with a larger root infection zone. Hence, NIN could play a negative regulatory role in determining nodule number, by restricting responsiveness to the root-sensitive zone (Marsh et al. 2007). Together, these data show that NIN is a key coordinator of bacterial entry and nodule organogenesis, and might integrate nutritional, hormonal and other signals into the nodulation process. Homology between the three identified NIN orthologues of different plants is restricted to six highly conserved domains, of which three have an unknown function. The other domains are predicted to encode transmembrane segments, a DNA-binding motif, as well as a C-terminal

protein-protein interacting region, implying that NIN acts as a TF (Schauser et al. 1999; Borisov et al. 2003). NIN might also share homology with Notch receptors that are proteolytically cleaved and generate both cytosolic and extracellular peptides. As the different peptides might have different signalling functions, they could be involved in both positive and negative regulatory roles (Marsh et al. 2007). Alternatively, the negative effect on the root-sensitive zone might be secondary because of lack of infection or primordium development.

6.4.3.3 ERN1

A TF involved in signalling at early stages of infection is ERN1. The protein is an AP2-domain-containing TF and has been identified as specifically binding to the NF box, a conserved NF-responsive element present in the promoters of *MtENOD11*, *MtENOD12* and *MtENOD9* (Andriankaja et al. 2007). The branching infection threads 1-1 (*bit1-1*) mutant, affected in *ERN1*, showed no or strongly reduced *ENOD11* induction when compared with wild-type plants, placing ERN1 in the NF signal transduction pathway (Middleton et al. 2007).

In the *bit1-1* mutant, rhizobial infection hardly progressed further than formation of infection foci. If ITs were formed, they would be arrested in the root hair cell or show complex and branched IT structures. More than 2 months after inoculation, this mutant formed small primordia, whereas wild-type plants developed numerous and healthy fixing nodules, implying that, besides a role during infection, ERN1 might also be involved in nodule formation. Indeed, introduction of the modified CCaMK in *bit1-1* never produced spontaneous nodules (Middleton et al. 2007).

Besides ERN1, two other ERN proteins were identified that bind to the NF box of the *ENOD11* promoter (Andriankaja et al. 2007). Both *ERN1* and *ERN2* induction required NFP and occurred only in root hairs upon NF treatments. Thus, ERN1 and ERN2 are possible downstream components of the epidermal NF signalling pathway. After transient expression in *Nicotiana benthamiana*, ERN-GFP/yellow fluorescent protein (YFP) proteins were localized to the nucleus, and both ERN1 and ERN2 acted as positive regulators, while ERN3 showed repression activity. Presumably, the three ERN proteins fine-tune NF-mediated gene expression in root hairs (Andriankaja et al. 2007).

6.5 Genes Involved in Infection, Formation and Development of Nodules

Rhizobial infection of the host plant involves IT initiation followed by IT growth through the root hair, penetration of several cortical cell layers and, finally, release of the rhizobia into the nodule primordium. After internalization, the bacteria differentiate into bacteroids and begin to fix nitrogen. All these processes are tightly

coordinated and mutants impaired at several stages of infection and nodule primordium development have been isolated and characterized.

Large-scale expression profiling experiments, using cDNA arrays or oligonucleotide chips, led to the identification of differentially expressed genes during nodulation. Based on these gene expression profiles, marker genes were identified for several stages of nodulation (Colebatch et al. 2002, 2004; El Yahyaoui et al. 2004; Mitra et al. 2004b; Lohar et al. 2006). Analysis of bacterial and plant marker genes during nodulation of mutant plant hosts might provide more information concerning the positioning of the affected genes in the nodulation process.

6.5.1 Marker Genes to Study Early Nodulation Stages

Examples of well-characterized markers of early nodulation responses in the epidermis are *ENOD* genes, such as *ENOD11*, *ENOD12* and *RIP1*, which encode a peroxidase and two proline-rich proteins that are transcribed during pre-infection and infection stages respectively (Journet et al. 1994, 2001; Cook et al. 1995). Also *MtN1* is associated with the infection process and can be used as a marker gene for this stage (Gamas et al. 1996, 1998). *ENOD20* is predicted to play a role in cell wall reorganization during IT growth and/or differentiation of the infected cells (Greene et al. 1998). A marker gene expressed in cortical cells immediately before IT penetration is *MtN6* (Mathis et al. 1999).

ENOD40 was shown to be induced at the onset of nodulation, and transcripts were localized in nodule primordium and pericycle cells. Because knocking down *ENOD40* results in reduced nodule numbers, *ENOD40* was proposed to play a role in nodule initiation and, thus, to be a good marker for early nodulation processes. However, transcripts were also present in the infection zone later in symbiosis. Thus, *ENOD40* might function in bacteroid differentiation. In *MtENOD40* silenced nodules, bacteria were released from the ITs and surrounded by symbiosome membranes, but they never developed into functional bacteroids and underwent premature senescence (Wan et al. 2007). Whereas the genes described above can be used as markers for infection and primordium initiation, *ENOD2*, *ENOD8* and *CCS52A* are markers for nodule development and differentiation, because they are induced in specific tissues and expressed prior to nitrogen fixation (Dickstein et al. 1993; Pringle and Dickstein 2004; Kuppusamy et al. 2004).

6.5.2 Genes Involved at Early Nodulation Stages

Several legume mutants are impaired in initiation and growth of ITs. Phenotypic characterization, together with expression analysis of molecular markers, can help to position the affected gene in the nodulation process. The first group of mutants show an arrest of infection in the root hairs or outer cortical cells. In the

M. truncatula Mtlin mutant, the number of ITs was reduced and they were all arrested in epidermal cells. The mutated gene is thought to maintain infection, but *MtN6*, involved in infection progression, is not induced in the mutant. The *ENOD20* gene, expressed during differentiation of nodule primordia, was induced similarly in *Mtlin* and wild-type plants, whereas transcriptional markers functioning in nodule differentiation (*CCS52*) and nodule morphogenesis (*ENOD2* and *ENOD8*) were not. Together, these data suggest that LIN also functions in nodule primordium differentiation (Kuppusamy et al. 2004).

Mutants of *L. japonicus* with defects in infection are *crinkle* (named after its crinkly trichomes) and aberrant localization of bacteria inside the nodule (*Ljalb*), which showed aberrant IT structures that arrested at the base of the epidermal cells and intercellular space respectively (Imaizumi-Anraku et al. 1997, 2000; Tansengco et al. 2003). In an *M. truncatula* mutant, infection was arrested downstream of the outer cortex; this altered nodule primordium invasion (*api*) mutant is blocked when the ITs invade the nodule primordium, leading to arrested nodule primordia filled with abnormal cortical infection structures that contain rhizobia (Teillet et al. 2008). Mutation of *API* mainly affected nodule primordium invasion at the inner cortex, but *API* is also thought to be involved at earlier stages of IT initiation and at later stages during IT growth, as was evidenced from the analysis of the few nodules that could overcome the invasion stop. Comparison of expression of the symbiotic markers *MtENOD12*, *MtN6* and *MtENOD8* confirmed these observations. *API*, which has not been cloned yet, is believed to act downstream of *ERN* and *LIN*, and upstream of genes involved in the further stages of development towards a nitrogen-fixing nodule (Teillet et al. 2008).

6.5.3 Genes Involved in Bacterial Differentiation and Nodule Development

Once bacteria are released in the plant cells, they differentiate into bacteroids. Meanwhile, the nodule primordia develop into zonated nodules with an active meristem and fixation zone. The *MtHAP2-1* gene is involved in nodule meristem function and encodes an HAP2-type TF subunit of the heterotrimeric CCAAT box-binding factor complex. Transcripts were present mainly in the meristematic zone of young nodules, and diminished in functional nodules. RNAi of *MtHAP2-1* delayed nodulation and arrested nodule growth at 8–10 dpi, resulting in spherical nodules without an active meristem. The meristematic cells had abnormal shapes and accumulated vacuoles. Also, bacteria were hardly released from ITs and nitrogen fixation did not occur. The knock-down phenotype suggested a role of *MtHAP2-1* in nodule development by controlling meristem persistence and, maybe, bacterial release. Interestingly, this gene is post-transcriptionally regulated by *miR169* degradation. *miR169* is expressed in the infection zone, adjacent to the meristem. Thus, miR169 might play an important role in defining a critical spatial

and temporal expression of *MtHAP2-1*, thereby contributing to the transition from meristematic to differentiated cells in nodules (Combier et al. 2006).

Mutants defective in meristem maintenance during nodulation and lateral root formation are the numerous infections and polyphenolics (*nip*) and *latd* mutants. These mutants both show reduced (and eventually arrested) growth of the primary and lateral roots. Also, nodule development stopped shortly after emergence. Hence, LATD might interfere with the maintenance of root and nodule meristems. In the small white nodules, aberrant ITs occurred that failed to release the bacteria, and lacked expression of bacterial and plant genes involved in bacteroid development or nitrogen fixation, implying that bacterial differentiation depends on nodule development, for which meristem maintenance and *LATD* expression are required (Veereshlingam et al. 2004; Bright et al. 2005). Abscisic acid (ABA) rescues the root meristem defects in *Mtlatd* but, due to the negative effect of ABA on nodule initiation, effects on nodule development could not be examined (Liang et al. 2007).

Another correlation between rhizobial differentiation and nodule meristem activity is found in the *Mtsym1* mutant, characterized by an altered infection process leading to the presence of two kind of non-fixing nodules. Some nodules were small, round, and had ITs limited to the outer root cortical cells, whereas in other elongated nodules, infection was normal and rhizobia were released but did not differentiate (Bénaben et al. 1995).

Finally, seven mutants defective in nitrogen fixation (*dnf1* to *dnf7*) had ITs that were indistinguishable from those in wild-type plants. Induction of rhizobial symbiosis gene promoters (*nodF*, *exoY*, *bacA* and *nifH*) and acetylene reduction activity were done to analyze the differentiation of the bacteria and their nitrogen-fixing capacity (Starker et al. 2006).

A first group of *dnf* mutants, containing *dnf1-1*, *dnf1-2* and *dnf5*, showed infection in the inner cortex, little or no nitrogen fixation, and no nitrogen fixation (*nifH*) expression or *MtN31* induction. These genes have been proposed to act upstream of other *DNF* genes. A second group of *DNF* genes comprises *DNF4* and *DNF7*, the mutants of which also lack nitrogen fixation and *nifH* expression but express *MtN31*. The third group of mutants, *DNF3* and *DNF6*, displayed reduced nitrogen fixation, but supported most *nifH* expression and express all nodulation-related genes tested (Starker et al. 2006).

6.5.4 Genes Involved in Nitrogen Fixation

Once bacteria are released from the ITs, enclosed by a symbiosome membrane, and differentiated into bacteroids, they can start to fix nitrogen via the nitrogenase complex. This nitrogenase complex contains several metal-sulphur clusters. *LjSST1* encodes a putative sulphate transporter located on the symbiosome membrane, and is presumably involved in transporting sulphate from the plant cytoplasm to the bacteroids. When sulphate transport was disrupted, as in the *Ljsst1* and *Ljsst2* mutants, sulphur content decreased and formation of nitrogenase was

inhibited, resulting in failure of symbiotic nitrogen fixation and early nodule senescence (Krusell et al. 2005).

The nitrogenase enzyme complex requires a low oxygen environment to convert N_2 to ammonia; in contrast, rhizobia are aerobic bacteria and need oxygen for respiration. To solve this contradiction, infected cells abundantly produce oxygen-carrying leghemoglobins. Symbiotic leghemoglobins were shown to bind free oxygen and thus lower the free O_2 tension. RNAi lines of *L. japonicus* with reduced transcripts of three symbiotic leghemoglobin genes had higher amounts of free oxygen in their nodules. The nodules developed normally, although they had a reduced cellular energy status, nitrogenase proteins were unstable, and nitrogen fixation was abolished, resulting in N starvation symptoms. These observations confirmed the necessity of leghemoglobin for nitrogen fixation in nodules (Ott et al. 2005).

An antisense approach revealed a function for *L. japonicus NDX1* and *NDX2* homeobox genes in nodulation (Grønlund et al. 2003). Silencing *Ljndx1* and *Ljndx2* resulted in disfunctional nodules. Although nodules appeared to be pink 3 weeks post-inoculation, nitrogen transport from nodule to root was reduced. More nodules were present on *Ljndx* antisense plants than on control plants, possibly because of the reduced N status of the plant affecting autoregulatory mechanisms. The nodules showed a reduced vasculature. Hence, NDX could participate in signalling pathways involved in differentiation of nodule parenchyma cells into vascular tissue. Another effect of silencing *NDX* was the lack of lenticels in the nodules and a modification of the nodule endodermis, which normally functions as an oxygen barrier. The NDX proteins might be involved in adaptation of the endodermis and epidermis to variable oxygen concentrations (Grønlund et al. 2003).

Carbon supply to the bacteroids is a strict requirement for the effective functioning of nodules. Symbiotic nitrogen fixation (SNF) depends primarily on the import of sucrose in the nodule. Sucrose synthase (SucS), cleaving sucrose in UDP-Glc and free fructoses, was shown to be essential for C supply, and plays a role in regulating the C metabolism and N fixation in nodules. Analysis in pea and *M. truncatula* revealed expression of the *SucS* gene in infected cells of the fixation zone, as well as in the meristematic region, prefixing zone, inner cortex, and nodule vasculature (Hohnjec et al. 2003). Antisense *SucS1* plants of *M. truncatula* and the pea mutant *rug4* showed less SucS activity, impaired SNF, and premature senescence (Gordon et al. 1999; Baier et al. 2007). Decreased SucS activity in nodules of *rug4* mutants lowered the contents of soluble proteins in nodules and of the leghemoglobin, but did not influence the expression of nitrogenase genes. However, nitrogenase activity was strongly reduced or was not measurable. Thus, SucS is required for the establishment and maintenance of an efficient nitrogen-fixing symbiosis, possibly because of the reduced flow of C towards the bacteroids or because of pleiotropic effects on other essential proteins or leghemoglobin (Gordon et al. 1999; Baier et al. 2007). It is generally accepted that dicarboxylic acids, such as malate and succinate, are the main respiratory C substrates for bacteroids and are transported via the symbiosome membrane to the bacteroids (Day and Copeland 1991).

In *M. sativa*, a Krüppel-like zinc finger protein, encoded by *Mszpt2-1*, is expressed in flowers and spontaneous nodules, as well as in vascular tissues in

roots and surrounding nodules. Antisense *Mszpt2-1* plants showed a Fix$^-$ phenotype. Functional analysis and expression data led to the hypothesis that this putative TF is involved in a cell-to-cell communication process between vascular tissues and the nitrogen-fixing zone, and might play a role in transporting metabolites related to the C/N metabolic status of the plant (Frugier et al. 2000).

Once the nitrogenase complex has converted N_2 into ammonium, ammonium is exported from the bacteroids into the cytosol of the infected cells, and assimilated into amino acids or ureids, which are exported from the nodule. NADH-dependent glutamate synthase (NADH-GOGAT) catalyzes together with glutamine synthetase the incorporation of ammonia into glutamate and is required for efficient SNF. Reduction of the level of NADH-GOGAT in alfalfa nodules resulted in nitrogen deficiency of plants grown under nitrogen-poor conditions. Impaired nitrogen assimilation and altered C/N ratios were caused by decreases in key enzymes for C and N assimilation, and amino acids and amides were reduced in the nodules (Cordoba et al. 2003). Asparagine, besides glutamine, the major assimilate from N fixation in temperate legumes, is synthesized by asparagine synthetase, which catalyzes amido transfer from glutamine to aspartate. On the other hand, tropical legumes, such as common bean or soybean, export ureides from nodules (Shi et al. 1997; Silvente et al. 2008).

6.6 The Latest Stage of Nodulation: Nodule Senescence

Aging or developmental senescence is a complex and highly organized process that has been intensively studied in many organisms and different plant organs. Also nodules are subjected to natural nodule senescence. At some point, nitrogen fixation in the nodule ceases and large-scale protein degradation occurs with, as final outcome, death of both bacteroids and nodule host cells. In pea, nitrogen-fixing activity in nodules is variable throughout the growth cycle. While nodules form a major C sink during the vegetative phase of the plant, during the pod filling stages, seeds have priority for C supply at the expense of nodules, possibly causing decreased nitrogen fixation (Voisin et al. 2003b). Indeed, for some crop legumes such as pea and soybean, nodule senescence coincides with pod filling (Swaraj and Bishnoi 1996). Exogenous application of nitrogen during pod filling improves both yield and seed protein content (Merbach and Schilling 1980). Moreover, in soybean, varieties exist with a delayed nodule senescence, indicating that the onset of the decay process is genetically controlled (Espinosa-Victoria et al. 2000). Hence, delaying nodule senescence might prolong the nitrogen fixation period and, thus, enhance seed protein content (Van de Velde et al. 2006).

Structural characteristics of natural nodule senescence in determinate and indeterminate nodules, and the involvement of hormones and reactive oxygen species (ROS) are currently being investigated. However, to date none of the signals have been identified that trigger the senescence phase in nodules, the signal transduction cascades, or regulatory functions controlling nodule senescence.

Natural nodule senescence comes with age and, consequently, starts in the oldest cells of the nodule. In indeterminate nodules, the process commences at the base of the nodule and progresses towards the apical zone, whereas in determinate nodules it begins at the centre of the spheric nodule and slowly spreads outwards (Puppo et al. 2005; Van de Velde et al. 2006).

Aging nodule tissues are easily distinguished by the pink-to-green colour shift, caused by the breakdown of the heme group of leghemoglobin that is abundantly present in fixing tissues (Swaraj and Bishnoi 1996). In determinate nodules of soybean, senescent nodule cells are characterized by a less electron-dense cytoplasm, numerous vesicles and a change in symbiosome structure, as well as membrane damage and increase in peroxisomes and mitochondria that form elongated structures (Puppo et al. 2005). In aging indeterminate nodules, the first signs of senescence appear in single infected cells located in the centre of the fixation zone and extend to the underlying cell layers towards the nodule cortex. Upon further development, a conically shaped senescence front migrates towards the nodule apex, eventually ending in a full senescent nodule (Pérez Guerra et al., unpublished data). A microscopic analysis in *M. truncatula* showed that the senescence zone can be subdivided into a younger and an older part. The cells in the younger senescence zone show signs of bacteroid degradation and resorption, while the host plant cells remain intact. In the older senescence zone, more senescent cells are found that are completely vacuolated, devoid of symbiosomes, and with signs of plant cell death, such as loosening of the plasma membrane from the wall. These observations imply two consecutive stages within developmental nodule senescence: a first stage characterized by bacteroid degradation and a more advanced stage during which plant cells start to decay and collapse. Hence, developmental senescence is a slow cell death process, presumably providing the plant time to resorb and recycle nutrients during organized breakdown of bacteroids and host cells (Van de Velde et al. 2006). The degradation of bacteria and plant cells, followed by transportation of nutrients and other compounds to the remainder of the plant, reflects transition of the nodule from sink towards source tissues (Van de Velde et al. 2006), as illustrated by the activation of genes encoding various catabolism-associated proteins. One group of cysteine proteinases (MtCP1-MtCP6) was shown to be upregulated during natural nodule senescence and produced in early degrading infected cells. Based on the presence of signal peptides, they were hypothesized to be targeted to the symbiosomes, where they might be involved in bacteroid degradation for nutrient remobilization. Analysis of the *MtCP6* promoter activity revealed that this gene can serve as a good marker for early stages of natural nodule senescence (Puppo et al. 2005; Van de Velde et al. 2006; Pérez Guerra et al., unpublished data). Also components of the Skp1-cullin-F-box-specific ubiquitin/26S proteasome pathway are observed during nodule senescence, indicating the importance of organized protein degradation. The onset of the catabolic stage was illustrated by the microscopically observed degradation of symbiosomes and plant cells (Van de Velde et al. 2006). Also genes encoding proteins involved in the transportation of nutrients, such as phosphate transporters, amino acids, and ATP-binding cassette proteins, were upregulated in senescent nodule cells (Van de Velde et al. 2006).

Parallel with a role of phytohormones in leaf and flower senescence, ethylene, gibberellins (GAs) and ABA are thought to interfere in developmental nodule senescence. ABA treatment of pea plants reduced nitrogen fixation and induced root nodule senescence (González et al. 2001). Based on transcript profiling experiments in *M. truncatula*, ethylene and GA3 might play a positive and a negative role respectively on developmental senescence in nodules (Van de Velde et al. 2006).

Besides hormones, ROS are also important in nodule senescence. Several reports demonstrate a decrease in antioxidant enzymes and in ascorbate and glutathione during nodule senescence. Differences in ROS levels seem to be more variable depending on nodule type, legume species or experimental conditions. Several hypotheses have been proposed but more experimental data are needed to elucidate the role of ROS during nodule senescence (Puppo et al. 2005).

Whereas initiation of natural nodule senescence is developmentally controlled, nodule senescence can also be induced prematurely by dark stress, nitrate fertilization, salt stress and drought stress (Sheokand et al. 1995; Escuredo et al. 1996; Swaraj et al. 2001; Gálvez et al. 2005). However, the progress of the natural and stress-induced senescence processes is very different. At first, the speed at which both processes evolve differs as stress-induced nodule senescence proceeds much faster than natural nodule senescence. Also, in contrast to the conical shape of the natural senescence zone, dark-induced nodule senescence migrates by a planar front. Senescing cells upon dark stress changed fast from a mature nitrogen-fixing status to a completely degraded content, but retained their rigid cell shape. In contrast to what was seen in senescing cells of naturally aging nodules, no pronounced vesicle mobilization was observed in the host cytoplasm and the peribacteroid membrane remained intact. These observations point to a general stress situation with rapid death of both the microsymbiont and the host cell (Pérez Guerra et al., unpublished data).

6.7 Hormones in Nodulation

As for all developmental processes in plants, nodule formation is ruled by different phytohormones that often act synergistically or antagonistically in a concentration-dependent manner. Hence, it is a considerable challenge to unravel how changes in hormones and hormone balances can interfere with the initiation, positioning, functioning and autoregulation of nodulation (Ferguson and Mathesius 2003).

6.7.1 Auxin

Auxin is synthesized in the shoot and is involved in various plant processes, such as cell division, cell differentiation, and formation of vascular tissue and lateral roots.

The hormone is transported to and throughout the root via an active acropetal transport mechanism mediated by the *AUX1* import and *pin-formed* (*PIN*) export carriers.

Proteome studies in *M. truncatula* revealed a high overlap in protein changes during early nodulation and after 24 h of auxin treatment of the root, suggesting that increased auxin levels can mediate early responses of the root upon inoculation and supporting a positive role for auxin during *M. truncatula* nodulation (van Noorden et al. 2007). The presence of auxin can be monitored in transgenic roots carrying an auxin-sensitive promoter of the soybean *GH3* gene fused to a reporter gene (pGH3:*uidA*). Spot inoculation of rhizobia or NFs on roots of white clover and vetch revealed that local inhibition of acropetal auxin transport resulted in an accumulation of auxin in the vasculature and cortical cells at the application spot, and a transient decrease in auxin between the inoculation site and the root tip (Mathesius et al. 1998; Boot et al. 1999). Auxin accumulation was observed in the nodule progenitor cells before the first cell divisions, and might be necessary for cell division and, thus, nodule initiation (Mathesius et al. 1998). This hypothesis is supported by the formation of spontaneous nodules upon application of synthetic auxin transport inhibitors, such as 1-naphthylphtalamic acid (NPA; Hirsch and Fang 1994).

In addition to NPA, natural auxin transport inhibitors, such as some flavonoids, inhibited polar auxin transport and caused local auxin accumulation (Mathesius et al. 1998). Roots deficient in chalcone synthase (CHS), a catalyzer of the first step of the flavonoid synthesis pathway, showed increased acropetal auxin transport upon inoculation and were unable to initiate nodule development, although normal bacterial infection occurred. Hence, flavonoids are good candidates to regulate auxin transport during nodule organogenesis (Wasson et al. 2006). Indeed, the flavonol kaempherol, the biosynthesis of which was stimulated by NFs, has been hypothesized to inhibit auxin transport (Zhang et al. 2009).

M. truncatula contains a family of at least five AUX1-like (*MtLAX*) genes that are closely related to the *AtAUX1* and might encode auxin import carriers. The genes were induced during early primordium formation, possibly to support auxin transport from the polar stream (which resumes at 2 dpi) towards the developing nodule (de Billy et al. 2001). Indeed, once cortical cell division is initiated, GH3:*uidA* expression is still present in the dividing progenitor cells, but strongly diminished in the surrounding cortical cells.

At a slightly later stage in nodule development, auxin was shown to be present at the base and periphery of nodules on white clover (Mathesius et al. 1998). Also, *MtLAX* gene expression and flavonoids colocalized at this position, where they might be necessary for the formation of nodule vasculature, redirecting auxin transport to allow new cell division and differentiation (de Billy et al. 2001; Wasson et al. 2006).

Both nodule formation and lateral root formation require high auxin levels to initiate cell division and to form new vascular tissues opposite the xylem poles. Moreover, in white clover, rhizobia have been shown to be able to hijack cortical

cells at LRBs and to form lateral root-associated nodules (LRANs) in a normally non-nodulating area of the root (Mathesius et al. 2000b).

The enzyme aldehyde oxidase (AO) is involved in auxin biosynthesis via oxidation of indole-3-acetaldehyde to form indole-3-acetic acid (IAA). The corresponding gene is highly expressed in the meristem and the infection zone of the nodules of *M. truncatula* and white lupin (*Lupinus albus*). Via AO, the plant can regulate nodule auxin synthesis, meristem growth and nodule development (Fedorova et al. 2005). Bacteria are also able to synthesize auxin. Auxin-overproducing derivatives of *S. meliloti* increased the number of indeterminate nodules on both *M. truncatula* and *M. sativa*, while increased synthesis of IAA in *R. leguminosarum* bv *phaseoli* had no effect on determinate nodule formation in common bean (Pii et al. 2007). In nodules in which IAA-overproducing bacteria reside, the presence of nitrogen oxide (NO) increased, suggesting an involvement of NO in the auxin signalling pathway to control indeterminate nodule formation (Pii et al. 2007). Several other studies also suggest that determinate and indeterminate nodules differ in auxin requirement. As such, in *L. japonicus*, no local inhibition of polar auxin transport occurred at the inoculation site (Pacios-Bras et al. 2003) and (iso)flavonoids, suggested as auxin transport regulators, were not required for nodule development in soybean (Subramanian et al. 2006). Also, *sunn*, a *M. truncatula* supernodulating mutant, has increased auxin levels at the nodulation initiation site, whereas the hypernodulating soybean mutant (*nts*) does not show increased auxin levels in the root (Caba et al. 2000; van Noorden et al. 2006).

6.7.2 Cytokinins

Cytokinins (CKs) are synthesized in the root tip and transported to the shoot via the xylem, and have a role in cell division, also during nodule development. One of the earliest experiments supporting a role of CKs in nodulation was the analysis of a *Rhizobium* mutant deficient in NF synthesis, but overproducing CKs due to the presence of a constitutively expressed *trans*-zeatin secretion gene (*tzs*) from *Agrobacterium tumefaciens*. Inoculation of alfalfa roots with this strain resulted in the formation of nodule-like structures, implying that increased amounts of CK can induce nodule formation (Cooper and Long 1994).

Exogenous application of CKs induced *ENOD40* expression in roots of white clover (Mathesius et al. 2000a). Also, *MtNIN* and other early nodulation genes have been shown to be induced by CKs (Fang and Hirsch 1998; Gonzalez-Rizzo et al. 2006). Moreover, CK application provoked other early nodulation responses, such as amyloplast deposition and cortical cell division (Bauer et al. 1996). Reduction of the CK content via overexpression of the CK oxidase (*CKX*) gene decreased nodule formation (Lohar et al. 2004).

Inoculation of transgenic *L. japonicus* roots containing the *ARABIDOPSIS RESPONSE REGULATOR 5* (*ARR5*):*uidA* construct, a marker for CK accumulation, revealed elevated CK amounts in dividing nodule progenitor cells (Lohar et al.

2004). Together, these data point to a positive role of CKs during the nodulation process.

A model has been proposed in which NFs cause locally enhanced CK levels, which are perceived by a CK receptor, followed by induction of cell division, leading to nodule primordia (Oldroyd 2007; Tirichine et al. 2007). The role of CKs in nodule primordia formation was shown unequivocally by analysis of plants with reduced CK receptor 1 (*MtCRE1*) expression in *M. truncatula*, and by studying *L. japonicus* mutants affected in the orthologous gene. *MtCRE1* is homologous to the *Arabidopsis AtCRE* and is involved in CK signalling. RNAi of *MtCRE1* resulted in inhibition of cortical cell division, absence of amyloplast deposition and reduced expression of early nodulins (Gonzalez-Rizzo et al. 2006). In *L. japonicus*, loss of function of the orthologue histidine kinase 1 (*LjLHK1*) in the hyperinfected1 (*hit1*) mutant abolished nodule primordia formation (Murray et al. 2007). A gain-of-function mutation, due to a single amino acid substitution in the CHASE domain of LjLHK1, in the spontaneous nodule formation 2 (*snf2*) mutant, resulted in a constitutively CK-independent activity leading to white nodules in the absence of *M. loti* (Tirichine et al. 2007). Both types of studies proved that CKs are sufficient and necessary for nodule organogenesis (Oldroyd 2007; Murray et al. 2007; Tirichine et al. 2007).

Bacterial invasion is thought to be independent of CKs because loss-of-function mutation in *LjLHK1* did not affect bacterial invasion of the root, although ITs developed highly elaborated structures that appeared to have lost growth direction (Murray et al. 2007). In contrast, silencing of *MtCRE1* blocked IT growth (Gonzalez-Rizzo et al. 2006), and *ARR5*:*uidA* was expressed also in curled and deformed root hairs (Lohar et al. 2004). It is possible that, besides cortical cell division, CK perception and signalling via MtCRE1 control also IT progression in the epidermis. However, IT growth might be arrested because of the inability of cortical cells to divide. These data indicate that CKs are essential for nodule formation and possibly control the cortical landscape to coordinate infection and cell division (Gonzalez-Rizzo et al. 2006). The latter would be in line with the observations that expression of the ERF TF required for nodule differentiation (*MtEFD*) could not be linked with infection per se. This TF was recently identified as an activating regulator of *MtRR4*, a CK primary response gene. As type-A response regulators, such as MtRR4, are thought to negatively control the CK pathway, its activation by MtEFD would explain the negative role of this TF at nodule initiation (increased and decreased nodule numbers upon RNAi and knock-out and overexpression respectively; Vernié et al. 2008).

Several observations suggest that CKs also play a role in later nodulation stages. As such, *MtCRE1* was expressed in the meristem of mature nodules (Lohar et al. 2006) and *MtEFD* expression was confined to the infection zone and seemed to be required for proper development of the infection and fixation zones, bacteroids and symbiosomes (Vernié et al. 2008). Based on these results, MtEFD probably participates in the control of a decreasing CK gradient from meristem towards underlying tissues via MtRR4 activation, and CKs are required for nodule meristem activity (Vernié et al. 2008).

6.7.3 Ethylene

Using ethylene precursors or inhibitors of ethylene synthesis or perception, various pharmacological studies have shown that, in several legumes, nodulation is inhibited by ethylene. One of the earliest observations was done by Grobbelaar et al. (1971), who established both reduction of nodule number and N fixation upon application of ethylene to roots of common bean. The same inhibitory effect was confirmed in pea and white clover (Drennan and Norton 1972; Goodlass and Smith 1979). Twenty years later, Lee and LaRue (1992) repeated these experiments and observed that the number of nodules was reduced by half, not as a consequence of a decreased IT formation but, rather, of an inhibition of IT growth in the epidermis or outer cortical cells. However, nodulation of soybean appeared to be insensitive to the hormone (Lee and LaRue 1992).

The negative role of ethylene on RHC infection was investigated in detail with various pharmacological approaches. Addition of 1-aminocyclopropane-1-carboxylic acid (ACC), the direct precursor of ethylene, effectively blocked nodulation in *M. truncatula* as well as in alfalfa, *L. japonicus* and siratro (*Macroptilium atropurpureum)*, but not in soybean (Guinel and Geil 2002). Application of the inhibitors of ACC synthase and of antagonists of ethylene action, aminoethoxyvinylglycine (AVG) and Ag^{+} ions respectively, increased the number of nodules (Nukui et al. 2000; Oldroyd et al. 2001). By growing *M. truncatula* on agar plates with different concentrations of ACC or AVG, Oldroyd et al. (2001) concluded that the number of nodules and infection events increased with decreasing levels of ethylene. As the ratio of nodule number to infection events did not change at different ethylene levels, these findings suggest that ethylene inhibits nodulation before or at the moment of IT initiation (Oldroyd et al. 2001).

Vetch has a thick, short root phenotype (Tsr) when inoculated and grown in the light, with increased number of root hairs, delayed nodulation and occurrence of nodules at sites of lateral root emergence (rather than on the primary root). This phenotype is suppressed by addition of AVG (Zaat et al. 1989). Later, cell biology revealed that the phenotype was caused by a swelling of cortical cells because of reorientation of the microtubuli. An excess of ethylene triggered by NFs under light conditions inhibited nodulation, probably by preventing the formation of pre-ITs in cortical cells (van Spronsen et al. 1995). Indeed, AVG rescued the Tsr phenotype in *L. japonicus*, but not in common bean where cytoplasmic bridges are not formed (van Spronsen et al. 2001).

Besides pharmacological experiments, the negative role of ethylene on nodulation was confirmed by the analysis of mutants and various transgenic plants. *L. japonicus* roots that ectopically expressed a dominant-negative version of the *Cucumis melo* (melon) *ERS1/H70A* ethylene receptor were ethylene insensitive, and had an increased number of ITs and nodule primordia after inoculation (Nukui et al. 2004). Similarly, introducing the dominant-negative ethylene receptor of *Arabidopsis* into *L. japonicus* showed an increasing number of nodules, proportional to the varying levels of ethylene insensitivity in independent transgenic lines

(Guinel and Geil 2002). Moreover, the *Mtskl* mutant, which is defective in ethylene perception because of a mutation in an orthologue of the *Arabidopsis* ethylene-insensitive 2 (*EIN2*) gene, was insensitive to ethylene and ACC in the triple response assay, and showed reduced leaf and petal senescence and increased root length respectively. Upon inoculation, an increased number of persistent infections in the nodulation zone of the root was observed in these *skl* mutants, resulting in a sickle-shaped root because of the very high number of nodule primordia (Penmetsa and Cook 1997; Penmetsa et al. 2008).

A natural variant of AVG is rhizobitoxin, a potent inhibitor of ACC synthase produced by *Bradyrhizobium elkanii*. Synthesis of the rhizobitoxin can possibly suppress the ethylene biosynthesis of its host plant siratro and so enhance nodulation (Yuhashi et al. 2000). Also, genes encoding ACC deaminase, which degrades the immediate precursor of ethylene, are present in some strains of rhizobia. It is possible that both gene products enable the bacteria to reduce the ethylene level in the host legume, thereby decreasing its inhibitory effect and facilitating infection (Ma et al. 2004). The inhibitory effect of ethylene on root hair infection was further analyzed by investigating the role of ethylene in root hair growth and NF signalling.

Besides influencing IT growth, ethylene also interferes with NF signalling before or at the level of Ca^{2+} spiking. Indeed, in *M. truncatula*, expression of the earliest nodulation genes, *rip1* and *ENOD11*, is reduced by application of ACC. Moreover, ACC reduces the number of root hair cells that respond to NFs with Ca^{2+} spiking and, in AVG-treated wild-type plants, more root hairs responded to NFs with Ca^{2+} spiking. Also, ACC treatment increased the NF threshold concentration needed for induction of Ca^{2+} spiking, and the periodicity of Ca^{2+} spiking in the ethylene-insensitive *skl* mutant increased (cf. diminished frequency of spikes; Oldroyd et al. 2001). Taking these data together, ethylene regulates a component of the NF signalling pathway at or upstream from Ca^{2+} spiking (via shortening of the Ca^{2+} spike period), and defines sensitivity of the plant towards NFs. Besides ethylene, also JA regulates Ca^{2+} spiking and sensitivity towards NFs with a similar, although opposite effect on the spike period (Sun et al. 2006).

In addition to an effect on infection and NF signalling, ethylene has also been shown to be involved in nodule primordium formation and positioning (Lee and LaRue 1992; Heidstra et al. 1997). The pea mutant *Pssym16* arrested IT growth in the inner cortex and inhibited further development of nodule primordia. This mutant has short internodes, few and thick lateral roots and reduced chlorophyll content, all pointing towards oversensitivity to ethylene, as confirmed by the restoration of the phenotype after addition of ethylene response inhibitors (Guinel and Sloetjes 2000).

In situ hybridization revealed the presence of ACC oxidase transcripts and, hence, ethylene production, opposite the phloem poles. In ethylene-insensitive mutants or transgenic plants, nodule formation extends towards the phloem poles (Lee and LaRue 1992; Heidstra et al. 1997; Guinel and Geil 2002; Penmetsa et al. 2003). Thus, together with auxin, CK and the stele factor uridine (Smit et al. 1995), ethylene contributes to nodule positioning opposite proto-xylem poles.

The role of ethylene in water-tolerant nodulation has been intensively investigated in the tropical legume *S. rostrata*. As diffusion of ethylene in water is much slower than in air, flooding causes an accumulation of ethylene that inhibits RHC infections in zone I of susceptible root hairs. Water-adapted legumes have found a way to circumvent this ethylene effect via crack-entry invasion in LRB nodulation (Goormachtig et al. 2004a, b). Ethylene is involved in intercellular infection pocket formation by induction of lesions and cell death (D'Haeze et al. 2003).

In addition to the infection mechanism being governed by growth conditions in *S. rostrata*, the type of nodules is also influenced by growth conditions. In a well-aerated environment, *S. rostrata* forms indeterminate nodules, whereas upon flooding, roots carry determinate nodules in which the meristematic activity disappears at a very early stage. Thus, ethylene is an important player in determining both infection mechanism and nodule type (Fernández-López et al. 1998).

6.7.4 Gibberellins

Gibberellins (GAs) comprise a large group of more than 130 diterpenoid carboxylic acids, four of which, GA1, GA3, GA4, and GA7, have an intrinsic growth-promoting activity. Little is known about the role of these hormones during nodulation. Higher levels of GAs have been recorded in nodules than in root tissues of various legumes. Several rhizobial strains produce GAs, but whether they contribute to the elevated level present in nodules is unknown (Ferguson and Mathesius 2003). Another indication for a role of GAs during nodulation is the nitrogen-sensitive formation of nodule-like structures from pericycle cell divisions upon exogenous application of GA3 on roots of *L. japonicus*, where GAs promoted cell division, necessary for nodule organogenesis (Kawaguchi et al. 1996).

GA-deficient mutants of pea showed reduced root development, fewer nodules and, in some cases, the phenotype was restored by exogenously supplied GAs. Exogenous application of low concentrations of GAs in wild-type pea stimulated nodule formation, whereas high concentrations became inhibitory for both mutants and wild-type plants, indicating a concentration-dependent role for GAs in nodule development (Ferguson et al. 2005).

A role for GAs has been established in the intercellular invasion process of LRB nodulation in *S. rostrata*. Transcripts of an enzyme of the GA biosynthesis pathway, *SrGA20ox*, accumulated upon NF treatment in cells associated with invading bacteria adjacent to intercellular infection pockets and ITs. Pharmacological approaches have shown that GAs are necessary for initiation of intercellular invasion. GAs were needed for nodule primordium formation in the cortex and for the establishment of nodule meristems. In zonated developing nodules, *SrGA20ox* transcripts were localized in cell layers at the meristem-to-infection zone transition, suggesting common features between nodule and shoot meristem formation (Lievens et al. 2005). Like ethylene, GAs inhibited RHC invasion in *S. rostrata* (Lievens et al. 2005).

6.7.5 Abscisic Acid

Initially, ABA was thought to inhibit nodulation because exogenous application to roots of pea and soybean results in a diminished number of nodules. Also, addition of abamine, an inhibitor of endogenous ABA formation, increases nodulation, thus supporting the hypothesis that ABA negatively regulates nodule numbers (Ferguson and Mathesius 2003; Suzuki et al. 2004). Additionally, ABA treatment on roots of white clover inhibited root hair deformation (Suzuki et al. 2004) and abolished both NF-induced Ca^{2+} spiking and *ENOD* expression (Ding et al. 2008).

In addition to physiological experiments, introduction of the *Arabidopsis abi1-1* allele, a dominant suppressor of ABA signalling, in *M. truncatula* enhanced NF-induced gene expression and increased nodule numbers. These results suggest that ABA can inhibit NF signalling at or above Ca^{2+} spiking. Whereas ethylene and JA influence NF signalling at a similar level, acting combinatorially, ABA is considered to act independently. A mutant sensitive to ABA 1 (*sta1*) showed reduced nodulation. In contrast, ABA was less effective in suppressing NF-induced Ca^{2+} spiking, meaning that *sta1* has a reduced ABA sensitivity towards NF signalling and that STA appears to specifically regulate ABA responses at different stages of the nodulation process (Ding et al. 2008). ABA treatment of the *snf2* mutants, carrying the gain-of-function *LHK1* mutation, abolished spontaneous nodulation and reduced CK induction of *ENOD40* and *NIN* in wild-type plants, suggesting that ABA can suppress CK responses in the cortex during nodule development (Ding et al. 2008). Taken together, ABA might exert a dual role in nodulation: restriction of nodule number by interference with NF signalling and positive regulation of nodule development via interference with CK in signalling pathways (Ferguson and Mathesius 2003; Ding et al. 2008). A possible third role of ABA might be at the onset of nodule senescence, when the ABA level increases a second time (Ferguson and Mathesius 2003).

6.8 Autoregulation

Although engaging into a nitrogen-fixing symbiosis renders the plant independent of available soil nitrogen, a high amount of energy must be invested by the plant for nodule development and functioning because the microsymbionts need to be provided with C sources. During the vegetative phase of the host plant, developing nodules comprise the largest C sink for the plant, while at flowering, most of the C and nutrient sources are relocated to seed filling (Voisin et al. 2003a). Hypernodulators of pea, bearing an excess of nodules, often show restricted shoot and root growth, while nitrogen fixation was never higher than in wild-type plants (Bourion et al. 2007). Hence, legume plants developed control mechanisms to equilibrate the level of nitrogen fixation, depending on the plant needs and the nitrate availability in the soil. Several of such control mechanisms exist and they act at different levels.

A first level of nodulation control is a systemic mechanism, the so-called autoregulation of nodulation (AON). Upon nodule organogenesis, a root signal is generated, transported to and perceived by the shoot, after which a shoot-derived autoregulation signal inhibits further nodulation in the root (Nishimura et al. 2002a).

Application of NFs to one root in a split-root assay of vetch caused diminished nodulation on the second root, suggesting that NFs can partly induce AON even in the absence of rhizobia (van Brussel et al. 2002). Also, split-root experiments with rhizobia strains deficient in NF production suggest that autoregulation in *L. japonicus* might be induced by NFs (Suzuki et al. 2008). However, additional unknown factors are probably needed to induce the full AON response.

The autoregulation shoot signal is presumably synthesized mainly in the leaf, because defoliation of soybean results in an increased number of nodules and more nodules occur on adventitious roots of excised leaflets of the hypernodulating mutant than on those of the wild type (Sato et al. 1997; Sheng and Harper 1997). The AON signal has been proposed to affect the infection efficiency, in split-root experiments of *L. japonicus* (Suzuki et al. 2008).

Several mutants deficient in AON have been identified, carrying many nodules all over the root system. The affected genes have been identified as hypernodulation aberrant root formation (*har1*) in *L. japonicus*, *nark* or nitrogen-tolerant symbiosis 1 (*nts1*) in soybean, symbiosis29 (*sym29*) in pea and super numeric nodules (*sunn*) in *M. truncatula* (Krusell et al. 2002; Nishimura et al. 2002a; Searle et al. 2003; Oka-Kira et al. 2005; Schnabel et al. 2005). Both *sunn* and *har1* mutants showed retarded root growth in the absence of rhizobia, but only *har1* had increased lateral root numbers (Penmetsa et al. 2003). Grafting experiments between these mutants and wild-type plants indicated that the shoot is responsible for the hypernodulating phenotype (Nishimura et al. 2002a; Searle et al. 2003).

The shoot is an important source of the plant hormone auxin. Auxin is transported from the shoot to the root, and it is a main regulator of nodulation. Auxin loading in the root of *M. truncatula* was enhanced in *sunn* mutants, compared to that of wild-type plants (van Noorden et al. 2006). At the onset of AON, auxin loading from the shoot to the root decreased in wild-type plants, whereas inoculation of *sunn* mutants failed to reduce auxin loading, suggesting that inoculation reduces auxin transport from the shoot to the root, thereby inhibiting further nodulation (van Noorden et al. 2006). Hence, auxin content in the root can be seen as a determinant of the total number of nodules. Auxin levels did not differ between the wild type and the *nts* hypernodulation mutant roots, implying different requirements for auxin in formation of indeterminate and determinate nodules (Caba et al. 2000; van Noorden et al. 2006).

The genes mutated in the hypernodulators encode the orthologous proteins LjHAR1, GmNARK, PsSYM29 and MtSUNN, which are RLKs with an extracellular leucine-rich repeat (LRR), a transmembrane domain, and a serine/threonine kinase domain (Nishimura et al. 2002a). The *Arabidopsis* gene with the highest similarity to the four genes is *CLAVATA1* (*CLV1*), which is expressed in the shoot apical meristem and encodes a protein that controls meristem identity by binding to the

CLV3 peptide after complex formation with AtCLV2 (Clark et al. 1997). Based on structural similarity of the proteins, it is possible that HAR1 and its orthologues also bind small peptides, but the search for their identity is still ongoing. Also, several phytohormones, such as brassinosteroids, JA and salicylic acid or its derivatives, are regarded as potential AON signalling candidates for the autoregulation signal, based on their systemic action in various induced disease resistance mechanisms (Oka-Kira and Kawaguchi 2006). Experiments in which the consequences of foliar hormone application on nodulation are examined might elucidate their role in AON. For example, foliar application of methyl jasmonate that suppresses nodulation in wild-type *L. japonicus*, and even hypernodulation of the *har1* mutants, suggests that JA and/or its related compounds might play a role in AON (Nakagawa and Kawaguchi 2006).

A different type of hypernodulator is the *klavier* mutant of *L. japonicus* that showed, besides a shoot-controlled supernodulation phenotype, also non-symbiotic phenotypes, such as convex leaf veins, delayed flowering, and dwarfism, phenotypes seen also in *clv1* mutants and in the pea mutants *sym28* and *nod4*. Further characterization of KLAVIER, SYM28 and NOD4 might reveal a new regulator controlling both shoot and nodule meristems (Oka-Kira et al. 2005; Oka-Kira and Kawaguchi 2006).

In addition to shoot-dependent hypernodulators, presumably impaired in perception of the root-derived signal or in transduction of the signal, root-regulated autoregulation mutants have also been identified, such as *nod3* of pea and root-determined nodulation (*rdn*) in *M. truncatula*, but the genes affected are still unknown (Oka-Kira and Kawaguchi 2006). The hypernodulation phenotype of a too much love (*tml*) mutant of *L. japonicus* was also root dependent and *TML* was proposed as a factor participating in the perception or transduction of the AON shoot signal (Magori et al. 2009).

Nodulation is also controlled locally by restriction of the number of efficient infections in the sensitive root zone. In wild-type plants, the number of ITs that grow into the cortex is low in comparison to the number of initiated infection events (Vasse et al. 1993). Ethylene might be involved in this process: upon infection with rhizobia, ethylene production is induced, which inhibits infection and blocks the growth of existing ITs (Penmetsa and Cook 1997; Oldroyd et al. 2001). *Mtskl* indeed showed an increased number of successful infection events.

Both ACC application and rhizobia inoculation cause changes in ethylene levels in the root, as well as decreased auxin transport. These effects were not observed in the *Mtskl* mutant, indicating that long-distance auxin transport is affected by ethylene concentration changes in the root (Prayitno et al. 2006).

Another hypernodulating mutant, *Ljastray*, not only showed an increased number of nodules, because the nodulation zone is not restricted, but also nodulated earlier than wild-type plants and its nodulation was light insensitive (Nishimura et al. 2002c). The mutant has a pleiotropic phenotype and its name is inspired by its agravitropic response. The mutated gene is homologous to *AtHY5* and was identified as bZIP and RING-finger protein (*LjBZF*), encoding a putative transcriptional

regulator with a zinc-finger domain, acidic region, casein kinase II (CKII) phosporylation site, and a bZIP domain. LjBZF might function as a negative regulator of nodulation (Nishimura et al. 2002b). The C-terminal half, containing the CKII and bZIP domain, is highly conserved between HY5 and BZF, whereas the N-terminal part is not. The N terminus, including the RING-finger domain and acidic region, shows high identity with the proteins STF1 and VFZBIPZF of soybean and broad bean respectively. The *Athy5* mutant shows enhanced lateral root initiation, whereas the *astray* mutant improved nodule initiation. Hence, motifs in the N region have been proposed to cause the different effect in *Arabidopsis* and *L. japonicu*s (Nishimura et al. 2002b).

6.9 Tools to Study Nodulation in Legumes

6.9.1 Genome and Sequence Analysis

M. truncatula is diploid and has 16 chromosomes, with euchromatin on the chromosome arms and heterochromatic DNA localized mostly at centromeres and pericentromeres. By means of fluorescent in situ hybridization, bacterial artificial chromosomes from the euchromatic gene-rich region could be selected for sequencing (Young et al. 2005). Localization of the insert clones via map resources that are available in *M. truncatula* (mtgenome.ucdavis.edu) and *L. japonicus* (www.kazusa.or.jp/lotus) allowed creation of (nearly) contiguous assemblies of genome sequences that comprise almost all of the euchromatin, the so-called gene space. The gene space of *M. truncatula* and *L. japonicus* is 270 and 230 Mbp respectively, with a gene density of 1 gene/6.7 kb and 1 gene/6.3 kb respectively, assuming a total of 35,000 to 40,000 genes (Young et al. 2005). Sequencing of both *M. truncatula* and *L. japonicus* is in progress and genome sequences are publicly available at www.medicago.org/genome and www.kazusa.or.jp/lotus, respectively. The clone-by-clone sequencing of *M. truncatula* is in process as a collaborative effort between the University of Oklahoma, the Samuel Roberts Noble Foundation, the United States National Science Foundation (NSF), the European Union 6th Framework Grain Legume Integrated Project (GLIP) programme, and other laboratories. In parallel, the genome of *L. japonicus* is being sequenced by the Kazusa DNA Research Institute in Japan.

Soybean is considered one of the major legume crops. Despite its polyploidy and complex genome, a profound understanding of the soybean genome organization and evolution is required for successful breeding and biological research (Stacey and VandenBosch 2005). While genome sequencing by a tri-agency group [NSF, United States Department of Energy (USDOE), and United States Department of Agriculture (USDA)] is in progress (Jackson et al. 2006), the sequencing of expressed sequence tag (EST) libraries has also been initiated in 1998 by The Public Soybean EST Project, and microarray and serial analyses of gene expression are underway (http://soybase.org).

Gene prediction programmes and comparison with EST databases has led to the annotation of pseudochromosomes. Currently, more than 25,000 genes have been predicted via the International *Medicago* Genome Annotation Group (IMGAG) and are waiting to be analyzed. Another application of the genome sequencing are tiling arrays, comprising oligonucleotides covering the entire sequence of pseudochromosomes, which can be used for gene identification, comparative genome hybridization (CGH), and chromatin immunoprecipitation (ChIP) on chips.

6.9.2 Transcriptomics

Large-scale sequencing of ESTs complements the genome sequencing projects in supporting functional genomics, because it identifies large gene collections and is a source for genome-wide tools such as DNA microarrays for transcriptome analysis. ESTs give information about gene structure, alternative splicing and in silico expression patterns, and are collected in the *Medicago* EST Navigation System (MENS; Journet et al. 2002; http://medicago.toulouse.inra.fr/Mt/EST/) and Dana-Farber Cancer Institute *Medicago* Gene Index (DCFI; Lee et al. 2005; http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=medicago), which is the successor of the *Medicago* Gene Index from The Institute for Genome Research.

The *L. japonicus* EST information and transcriptome data are provided by the *Lotus* EST database at http://www.kazusa.or.jp/en/plant/lotus/EST. In all, more than 200,000 ESTs have been collected from cDNA libraries constructed after various plant treatments, from different organs and at diverse stages of nodulation. Comparison of the legume EST data with non-legume sequence data led to the identification of 2,525 legume-specific contigs, and in silico analysis of *M. truncatula* EST nodule libraries revealed 340 genes expressed solely during nodulation (Fedorova et al. 2002).

Although in-depth in silico studies may provide ample information for further research, the predictions should be checked thoroughly, via transcript profiling with microarray or related analyses. The GeneChip Medicago Genome Array contains more than 61,200 probe sets of which more than 50,000 are from *M. truncatula* ESTs and 8,305 from *S. meliloti* sequences. Including the probe sets for 1,850 *M. sativa* transcripts on the chip facilitates comparative study of closely related legume species (www.affymetrix.com/support/technical/datasheets/medicago_datasheet.pdf). Using the Affymetrix Medicago Gene Chip, a compendium or "atlas" of gene expression profiles for the majority of *M. truncatula* genes covering all its major organ systems (roots, nodules, stems, petioles, leaves, vegetative buds, flowers, seeds and seed pods) has been developed recently (http://bioinfo.noble.org/gene-atlas; Benedito et al. 2008; http://www.cebitec.uni-bielefeld.de/truncatulix/; Henckel et al. 2009).

6.9.3 Mutagenesis of Model Legumes

Whereas sequence information and gene expression data can help to unravel the function(s) of genes, the allocation of gene functions requires the phenotypic analysis of knock-out or knock-down plants. By forward genetics, mutagenized plant populations are screened for a defect in certain processes, after which the affected gene is identified by map- or transcript-based cloning. In reverse genetics, mutant collections are used as starting material to search for mutations in specific genes, after which the phenotype of the mutant plant is analyzed. An important source for these analyses in *M. truncatula*, *L. japonicus* and pea plants are the mutant collections generated by different mutagenesis approaches [ethyl methane sulfonate (EMS), fast-neutron radiation, and transposon insertions] that can be used for research based on either forward or reverse genetics.

Examples of such mutant collections are the Tnt*1* insertion mutant lines of *M. truncatula* R108 and A17 generated by the Noble Foundation and the European GLIP project. Tnt*1* is a tobacco retrotransposon that transposes in the genome by a “copy and paste” mechanism, involving an intermediate RNA reverse transcribed to cDNA and subsequently integrated into the genome. Tnt*1* has considerable advantages compared with other transposons, because it transposes efficiently in the *M. truncatula* genome upon transformation and regeneration and is stably integrated into the genome of growing plants (d’Erfurth et al. 2003; Tadege et al. 2008). At present, 7,600 independent mutated lines of *M. truncatula* have been generated, representing an estimated 190,000 insertion events. Cloning of the tagged genes in these lines is relatively easy because flanking sequence databases are available. Currently, databases of flanking sequence tags (FSTs) are being constructed for reverse screening (J. Murray and M. Udvardi, personal communications). However, to obtain a genome-wide covering of insertion mutants, more mutant lines should be generated (Tadege et al. 2008).

EMS treatment causes point mutations (mostly G/C-to-A/T transitions) in the plant genome, providing a series of allelic mutants for functional analysis. The mutant populations can be used for forward and reverse genetics. Once the phenotype is identified, positional cloning of the responsible gene requires mapping of the mutation and identification of closely linked molecular markers that can be sequenced and compared to wild-type sequences. A forward screen on the progeny of 4,190 EMS-mutagenized M1 *L. japonicus* plants was carried out to identify novel mutants. A database with information on individual mutant plants is accessible at http://data.jic.bbsrc.ac.uk/cgi-bin/lotusjaponicus (Perry et al. 2003). An EMS-generated mutant population has been screened by reverse genetics through the Target-Induced Local Lesions In Genomes (TILLING) procedure. This PCR-based technique involves the formation of heteroduplexes between PCR products of the wild type and mutated DNA fragments, and mismatch cleavage by the endonuclease CEL1 (Henikoff et al. 2004). A TILLING resource for screening 4,500 M2 *M. truncatula* plants is available at the Institut National de la Recherche Agronomique (INRA, Dijon and Montpellier) and John Innes Centre (JIC,

Norwich). In addition, the JIC and the Sainsbury Laboratory provide TILLING application on 4,808 M2 *L. japonicus* EMS mutants (Perry et al. 2003), and INRA (Evry and Dijon) developed a collection of 4,704 pea mutants within the European GLIP (http://www.sciencedaily.com/releases/2008/02/080225213703.htm).

The advantages of EMS are its applicability to any crop, independent of transformation capacities or genome size. Moreover, series of allelic mutations can be obtained that display a range of phenotypes to be used for more detailed functional analysis. As EMS gives rise to point mutations that generally have no or little effect on the activity of the mutated protein, alternative mutagenesis and reverse screening techniques are needed for fast identification of knock-out mutants.

A frequently used alternative to create mutagenized populations is fast-neutron radiation; it can cause chromosomal rearrangements, under which DNA deletions form a few base pairs up to more than 30 kb. Fast-neutron-mutated lines can be analyzed by forward as well as reverse genetics. To identify the deleted genes in a forward genetics approach, microarrays are used to look at global gene expression or genomic differences (Tadege et al. 2005). For reverse screening of *M. truncatula* mutants, a de-TILLING technique is currently being developed by JIC and the Noble foundation. This technique combines restriction enzyme suppression and poison primer suppression to improve PCR efficiency by eliminating the PCR amplification from wild-type sequences, abundantly present in pooled DNA. JIC and the Noble foundation currently have DNA of 70,000 M1 *M. truncatula* mutant lines available for reverse screening via de-TILLING (C. Rogers and R. Chen, personal communications).

6.9.4 From Model Legume to Crop Legumes

Collectively, setting up these tools for functional genomics will allow rapid progress in understanding the molecular basis of nodule formation in model legumes. Moreover, conserved genome structure (synteny) studies between several (model) legumes have provided insights into the organization and evolution of legume genomes, and the differences and similarities with other plant families. With a model legume as reference, comparative genomic tools have already formed a bridge towards crop legumes to predict and isolate several genes required for root symbiosis. Genetic, genomic and molecular tools available for model legumes can deliver information about the molecular basis of nodulation, and also help to transfer these data to important crop species (Ané et al. 2008).

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Chapter 7
Tuber Development

W.L. Morris and M.A. Taylor

7.1 Introduction

Tubers are short, thickened, mostly underground stems. They bear minute scale leaves, each with a bud that has the potential for developing into a new plant. The term is also used imprecisely but widely for fleshy roots or rhizomes that resemble tubers (e.g. the "tubers" of the dahlia are actually tuberous roots). Throughout the history of humankind, tubers have had an important role to play in food availability. The migration of early hominids from the tropical rainforest to the savannah, thought to have occurred after *Ardipithecus ramidus* (ca. 4.4 million years B.C.), resulted in food acquisition becoming more critical, and "feast and famine" cycles in food availability were common (Kays and Paull 2004). Tubers comprised significant components of the diet and, importantly for hunter-gatherer societies, were available over extended periods of time due to their ability to be left in situ until needed. Even today, many indigenous populations display a remarkable knowledge of the general biology of the plant material they gather from the wild.

According to some sources, there are 23 cultivated plant species that produce tubers (http://www.uga.edu/rootandtubercrops/English/), including aerial yam, African yam, country potato, cush-cush yam, earth chestnut, false yam, hausa potato, Japanese artichoke, Jerusalem artichoke, potato, water yam, white Guinea yam and yellow yam. In modern agriculture, by far the most widely cultivated tuber-bearing species is the potato (*Solanum tuberosum* L.), with production of 315 million tonnes worldwide (http://faostat.fao.org). Potato is a crop fourth in terms of world production after wheat, rice and barley. However, potatoes produce more dry matter and protein per hectare than do the major cereal crops (Burton 1989). Yams are also an important crop, with an annual production of 39 million tonnes

W.L. Morris and M.A. Taylor
Plant Products and Food Quality, Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, UK
e-mail: mark.taylor@scri.ac.uk

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(http://faostat.fao.org). Their cultivation is particularly important in the humid and dry tropics of West Africa (Eka 1998). Yam is the common name for a number of species of the genus *Dioscorea*. The most commonly cultivated African yams are *Dioscorea rotundata* (white or Guinea yam) and *Dioscorea cayenensis* (yellow yam), whereas in Asia the most common yam is *Dioscorea alata* (water yam). Other tuber-bearing species listed are not widely cultivated; e.g. Jerusalem artichoke is not produced at a level sufficient to appear in annual production statistics monitored by the Food and Agriculture Organisation and, thus, is regarded by some as an underutilized resource (Kays and Nottingham 2007).

7.1.1 Tuber Composition and Nutrition

Tubers have been an important part of the diet of humankind throughout history. The chemical composition of potato has been well studied, reflecting its importance as a food crop. The nutrient content of potato depends on the genetic features of the cultivar and on the environment in which it is grown, although some generalizations can be made. Carbohydrates constitute approximately 75% of total dry matter and potatoes are a good source of protein, vitamins and dietary fibre (Storey 2007). An average (175 g) serving of potato provides over 40% of the Recommended Daily Allowance for vitamin C, approximately 30% of vitamin B6 requirement, 16% of vitamin B1 requirement and 16% of folate requirement, as well as potassium, iron and magnesium (Storey 2007). The flesh colour of potato tubers is due to the accumulation of two different classes of pigment. Whereas anthocyanin accumulation leads to red, blue or purple flesh colours (Hung et al. 1997), carotenoid levels determine whether the tuber flesh is white, yellow or orange (Nesterenko and Sink 2003). Research is only recently starting to address the factors that influence the levels of some of these nutrients but, as the demand for nutrient-rich food increases, these factors will become more important as breeding targets.

Data on the nutritional content of yams are available (Okwu and Ndu 2006; Huang et al. 2007). However, there is wide genetic and environmental variation within yam germplasm. The wide genetic variation observed constitutes a good basis for genetic improvement of yam. As with potatoes, yams are a good source of carbohydrate in the form of starch. The protein (mainly in the form of the storage protein discorin) content is approximately 10% of dry weight and yams are a source of minerals, particularly potassium, calcium and iron, and of vitamin C. Interestingly, some yam varieties are used traditionally for medicinal purposes, reflecting a complex phytochemical profile that remains to be fully explored. For example, the yam sapogenin diosgenin is a precursor for the hemisynthesis of progesterone, oestrogen and corticosteroids (Crabbe 1979).

A distinctive feature of Jerusalem artichoke tubers is that carbohydrate is stored in the form of inulin, rather than starch (Somda et al. 1999). Inulin is a mixture of fructose polymers, typically containing a much lower degree of polymerization than is the case for starch, and consisting of 2–70 fructose subunits (De Leenheer 1996).

There is a wide and increasing range of food uses for inulin that may stimulate interest in the wider-scale cultivation of Jerusalem artichoke. Apart from inulin, Jerusalem artichoke tubers are a good source of minerals, particularly iron, calcium and potassium. Vitamin C content is generally in the range 2–6 mg/100 g, which is higher than that in most potato cultivars. B complex vitamins and β-carotene are also present in Jerusalem artichoke tubers (Kays and Nottingham 2007).

7.1.2 Focus on Potato

Research effort is reflected to a large degree in the relative production of tuber-bearing species. Thus, our knowledge of potato tuber development greatly exceeds that of tuber development in other species. In potato, there is a wide range of genetic resources and the crop has long been subject to molecular and transgenic studies (Millam 2007). More recently, genomic resources have been developed, including large expressed sequence tag (EST) databases and detailed microarrays (http://www.tigr.org/tdb/sol/). The POCI 44000 feature microarray is the best microarray platform currently available to analyze global gene expression in potato (described in Kloosterman et al. 2008), representing 42,034 unigene sequences. Most potato microarray experiments performed to date have used the widely accessible spotted cDNA array produced by the Institute for Genomic Research (TIGR), which contains about 12,000 cDNA clones (http://www.tigr.org/tdb/potato/microarray_desc.shtml). It has been estimated that 35,000 genes are expressed in tomato (Van der Hoeven et al. 2002) and a similar number are probably expressed in potato. The progression of the potato genome sequencing project by the Genomics Sequencing Consortium (http://www.potatogenome.net), established with the objective of elucidating the complete sequence of the potato genome by 2009, will provide further impetus to understanding the biology of the potato tuber.

7.2 Potato Tuber Development

Potato tubers develop from lateral underground buds at the base of the main stem, which grow out diagravitropically to form specialized underground stems called stolons (Cutter 1978). On induction to tuberize, longitudinal stolon growth ceases and swelling growth occurs in the sub-apical region of the stolon (reviewed in Vreugdenhil and Struik 1989). Anatomical studies have described the changes in cell type, shape and growth pattern that result in tuber formation (Fig. 7.1). Initial expansion and radial cell division of cells in the pith and cortex, in combination with restricted longitudinal growth, is followed by cell division and enlargement in random orientations in the perimedullary region once the swelling has reached a diameter of 2 to 4 mm, continuing until the tuber reaches its full size (Xu et al. 1998). The molecular mechanisms that control tuber formation have been investigated in detail over the past few decades because the control of tuber number and

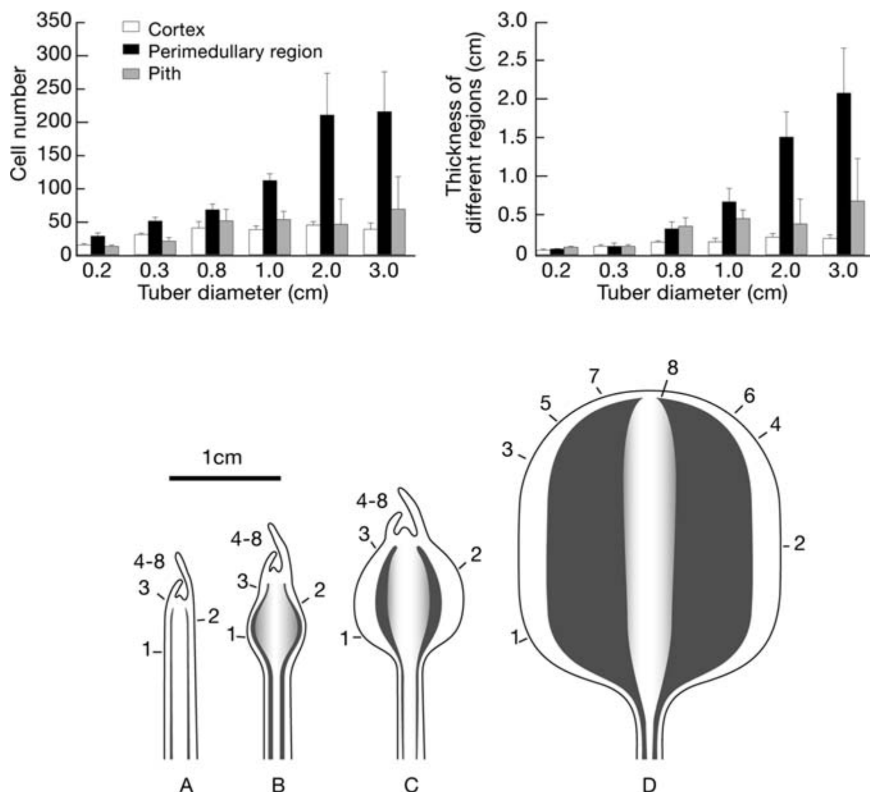

Fig. 7.1 Diagram of longitudinal sections through in vivo grown potato tubers, showing the morphology of the stolon and tuber and the thickening of the perimedullary zone (*dark-shaded area*). Positions of nodes are indicated schematically. The *numbers* indicate the nodes. Bar = 1 cm. *A* 0.2-cm stolon, showing the continuous vascular bundle. *B* 0.3-cm tuber, showing the growth of pith (*light-shaded area*). *C* 0.8-cm tuber, showing the onset of growth of the perimedullary region. *D* 2.0-cm tuber, showing the thickening perimedullary region (source: Xu et al. 1998, with permission from Oxford University Press)

size uniformity is probably the largest economic constraint of modern potato production (reviewed in Vreugdenhil and Struik 1989; Hannapel 1991; Ewing and Struik 1992; Jackson 1999; Fernie and Willmitzer 2001; Rodriguez-Falcon et al. 2006). Few poorly refined practices with dubious efficacy are available for manipulating this trait in the field. For example, seed tubers can be managed using heat/light, low temperatures, wounding and physiological age to influence stem numbers (Knowles and Knowles 2006; Struik et al. 2006). However, the effectiveness of these practices is limited. Appropriate seed spacing can help to ensure uniform tuber set and development (Bussan et al. 2007) but practices are not available for inducing synchronous tuber setting and, hence, ensuring uniformity in tuber size and, ultimately, a more uniform crop. One key problem is that all the

available approaches are empirical in nature and not designed specifically to act upon the basic mechanisms influencing the tuber life cycle (e.g. bud dormancy, apical dominance and tuberization).

7.2.1 Control of Tuber Initiation

The factors that determine when stolons differentiate into tubers have been studied extensively (reviewed in Vreugdenhil and Struik 1989; Hannapel 1991; Ewing and Struik 1992; Jackson 1999; Fernie and Willmitzer 2001; Rodriguez-Falcon et al. 2006). In general, tuberization is promoted by long nights, cool temperatures, low rates of nitrogen fertilization and the physiological age of the seed tuber. However, considerable genotypic variation exists in the response to day length and climate, reflecting the geographic origin of the germplasm. For example, wild Andean varieties such as *Solanum tuberosum* spp. *andigena* were originally cultivated in the highlands of South America near the equator, where day length remains close to 12 h throughout the year and temperatures are low at night. Such varieties tuberize poorly under the higher temperatures of lowland tropics or the long days of temperate zone summers and are considered to be obligate short-day plants (Van den Berg et al. 1995). In contrast, most cultivated potatoes are derived from Chilean landraces (*Solanum tuberosum* spp. *tuberosum*) adapted to longer summer days. During the development of modern cultivars, selection for tuberization under longer days has resulted in modern cultivars being independent of day length (Rodriguez-Falcon et al. 2006).

The strict short-day length requirement of Andigena varieties has led to the development of elegant experimental systems for studying the signals that result in tuberization. Early work demonstrated that the tuberization stimulus was perceived in the leaves, resulting in the production of a graft transmissible signal that is transported basipetally to the stolon tip where it induces tuberization (Gregory 1956; Jackson et al. 1998). The chemical nature of the stimulus, however, remains to be clarified (Suarez-Lopez 2005). In recent years it has become apparent that the photoperiodic control of flowering and tuberization encompasses elements with similar functions (Rodriguez-Falcon et al. 2006). For example, the photoperiodic control of tuberization requires phytochrome B (Jackson et al. 1996). Homologues of GIGANTEA, CONSTANS and flowering locus T, elements well characterized in the day-length control of flowering pathways of Arabidopsis and rice (Koornneef et al. 1991), are also implicated in the short-day pathway controlling tuberization in potato (Rodriguez-Falcon et al. 2006; Fig. 7.2). In Arabidopsis, expression of the transcription factor CONSTANS accelerates flowering in response to long days (Putterill et al. 1995). Constitutive over-expression of the Arabidopsis CONSTANS gene in potato results in a delayed tuberization phenotype (Martinez-Garcia et al. 2002).

Plant growth regulators have also been implicated in the control of tuber initiation. In particular, the role of gibberellins in this respect has become clearer in

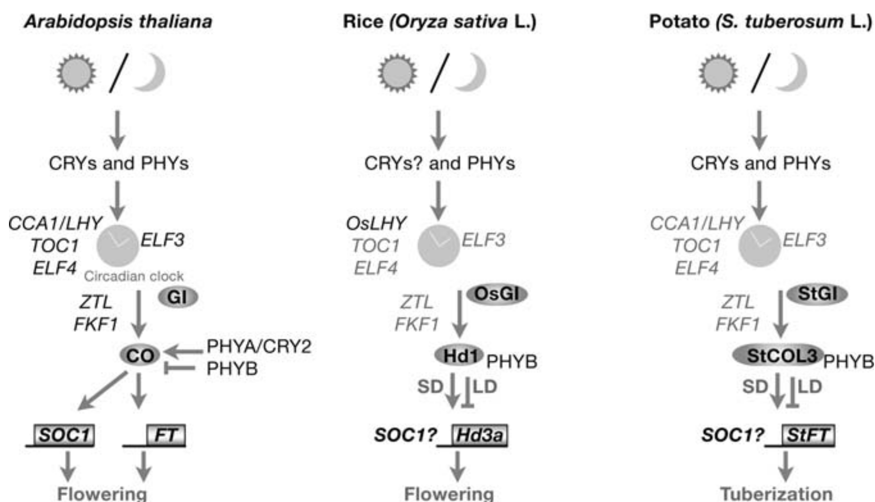

Fig. 7.2 Short-day (SD) pathway controlling tuberization in potato: conservation with the day-length flowering pathways in Arabidopsis and rice. The circadian clock is reset by phytochromes and cryptochromes and is the central component of the day-length measuring mechanism. The clock regulates abundance of GIGANTEA (*GI*), which positively regulates transcription of the nuclear zinc-finger proteins constans (CO) and Heading date 1 (Hd1). These transcription factors in turn regulate expression of flowering locus T (FT) and Heading date 3a (H3da), two RAF-kinase inhibitor-related proteins that strongly promote flowering. CO/Hd1 activity is regulated by light in a post-transcriptional manner. Whereas CO activates transcription of *FT* in the light, Hd1 appears to activate *H3da* expression in the dark but to negatively control expression of this floral integrator in the light. Interaction of Hd1 with phytochrome appears to mediate *H3da* repression in the light. Homologues of *GI* (*StGI*), *CO* (*StCOL3*) and *FT* (*StFT*) have been identified in potato and preliminary evidence indicates that they are implicated in tuberization control. *StFT* mRNA accumulation correlates with the peak of *StCOL3* transcript during the night, suggesting a light-reversed mechanism of regulation, as reported in rice. Components of the clock autoregulatory feedback loop such as *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*), *TIMING OF CAB EXPRESSION1* (*TOC1*) and *EARLY FLOWERING4* (*ELF4*) have not been characterized in potato. Genes involved in the light input to the clock, such as *EARLY FLOWERING 3* (*ELF3*) or the F-box kelch repeat proteins *FLAVIN-BINDING KELCH REPEAT F-BOX1* (*FKF1*) and *ZEITLUPE* (*ZTL*), also remain to be identified (source: Rodriguez-Falcon et al. 2006, with permission from Annual Reviews)

recent years. It has been demonstrated that there is a decrease in the concentrations of gibberellic acid (GA) in the stolon tip at the onset of tuberization of microtubers grown in vitro, resulting in the reorientation of microtubules and a shift in the plane of cell division. The regulation of GA concentrations may occur via transcriptional control (StBEL5, POTH1; Chen et al. 2004) or regulation may be brought about by altered sensitivity to GA (PHOR1; Amador et al. 2001). Transgenically manipulated changes in the expression levels of these genes affect the onset of tuberization. More recently, the up-regulation of a GA 2-oxidase gene early in the tuber initiation process, in the sub-apical region of the stolon, provides another mechanism whereby the GA concentration in this region is decreased (Kloosterman et al. 2007). Thus, it appears that there are two main pathways controlling the onset of tuberization,

namely a short day (or, more accurately, a long night)-dependent pathway and a GA-dependent pathway. It remains to be clarified how these two pathways interact. A genetic approach may provide the way forward, as already 11 quantitative trait loci (QTLs) affecting tuberization have been defined in reciprocal backcrosses between *S. tuberosum* and *Solanum berthaultii* (van den Berg et al. 1996). With the potato genome project underway and the development of high-throughput gene mapping platforms, the identification of genes underlying these QTLs is likely to occur in the next few years. It may then be possible to understand, at the molecular level, the adaptive changes that occurred in modern germplasm as the response to day length has been selectively modified. Furthermore, the goal of producing a crop that is more coordinated in tuber initiation may finally be attainable.

7.2.2 Changes in Carbohydrate Metabolism During Tuber Development

The major storage carbohydrate in potato tubers is starch, which typically accounts for 20% of tuber fresh weight (Storey 2007). Starch is synthesized from sucrose, produced in the leaves by photosynthesis. The conversion of photoassimilates to sucrose allows transport from source leaves to developing tubers via the phloem. The factors that are involved in the transfer of sucrose from photosynthetically active tissues to the phloem have been researched intensively and many of the details of these processes are understood (reviewed in Hofius and Börnke 2007). Of particular interest is the change in phloem unloading mechanism that occurs in the early stages of tuber development (Viola et al. 2001a). Prior to visible tuber development, the predominant pathway of phloem unloading is apoplastic. However, there is a switch to predominantly symplastic unloading in the early stages of tuber development. Concurrent with the pattern of unloading is a change in the way in which sucrose is cleaved into hexose. Prior to tuberization, sucrose is cleaved predominantly by acid invertase but the switch in the unloading mechanism is paralleled by an increase in sucrose synthase activity. The effects of its down-regulation in transgenic lines (Zrenner et al. 1995) demonstrated the importance of sucrose synthase in the development of tuber sink strength. These lines had reduced starch and protein content, as well as fewer tubers. Following sucrolysis, the products are metabolized to hexose-phosphates by the action of either UDP-glucose pyrophosphorylase, in the case of the sucrose synthase pathway, or hexokinase and fructokinase in the case of the invertase pathway (Fig. 7.3). As starch synthesis takes place in the amyloplast, the hexose phosphate precursor must be transported to this organelle. It is thought that glucose-6-phosphate is the substrate that is transported into the amyloplast for starch synthesis (Kammerer et al. 1998). In heterotrophic tissues such as tubers, ATP must also be imported into the amyloplast in order to drive the biosynthetic reaction. Glucose-1-phosphate and ATP are converted into ADP-glucose in a reaction catalyzed by ADP-glucose pyrophosphorylase, the first committed step in starch biosynthesis. Following this reaction,

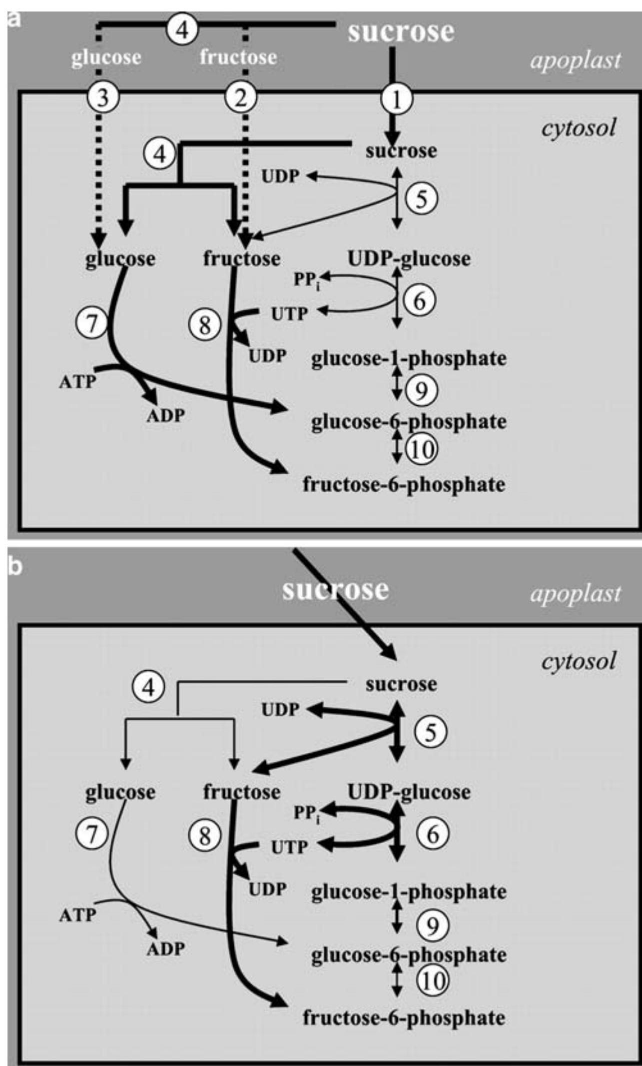

Fig. 7.3 The predominant route of sucrose unloading and subsequent mobilization. **a** Prior to tuber initiation. **b** During tuber enlargement. The *numbers* denote the following enzymes: 1, sucrose transporter; 2 and 3, hexose transporter(s); 4, invertase; 5, sucrose synthase; 6, UDP-glucose pyrophosphorylase; 7, hexokinase; 8, fructokinase; 9, phosphoglucomutase; and 10, phosphoglucose isomerase. The thickness of the *arrow* indicates the predominant flux (source: Fernie and Willmitzer 2001, with permission from American Society of Plant Physiologists)

starch biosynthesis is achieved by the action of starch synthases, branching and debranching enzymes (Kossmann and Lloyd 2000; Tetlow et al. 2004). It has been shown that the adenylate supply to heterotrophic amyloplasts has a strong influence on the levels of starch that accumulate. Over-expression of the amyloplastidial ATP:ADP translocator results in increased starch accumulation, whereas reduced

expression decreases starch yield (Tjaden et al. 1998). Furthermore, decrease in the activity of a plastidial adenylate kinase gene has a major effect on tuber metabolism, demonstrated by Regierer et al. (2002) using a transgenic approach. In some transgenic lines, starch yield increased by up to 60% and tuber yield by up to 39%. Additionally, free amino acid levels increased significantly.

7.2.3 Other Aspects of Metabolism—Sugar and Amino Acid Content

An important issue in tuber quality is the concentrations of carbohydrates other than starch, predominantly the reducing sugars (glucose and fructose) and the non-reducing disaccharide sucrose. Tuber sugar content is dependent on the variety and physiological status of the tuber, and the concentrations of sugars change during development and subsequent storage (reviewed in Sowokinos 2001). As tubers develop, they reach maturity when the ratio of sucrose to glucose and fructose reaches a minimum. Sugar concentrations are an important issue for processing because reducing sugars can undergo a Maillard reaction with the α-amino group of nitrogenous compounds, giving rise to browning in processed products (Schallenberger et al. 1959). Thus, the management of sugars at harvest and during storage is important to the potato industry (reviewed in Storey 2007). Tuber sucrose concentrations generally increase during storage at low temperatures (less than 4°C), although reconditioning of the crop by storage at higher temperatures for 2–6 weeks can reverse this process. Senescent sweetening occurs during prolonged storage and this accumulation of reducing sugar is not reversible.

The amino acid biosynthetic networks are complex and heavily regulated (Roessner-Tunali et al. 2003), although most of the biosynthetic genes have been cloned. The contribution of amino acids synthesized in the tuber and amino acids imported from leaves remains to be fully resolved (Fischer et al. 1998), but clearly amino acid transporters could have a key role (Koch et al. 2003). Some evidence suggests that the concentrations of the umami amino acid glutamate may impact on tuber taste (Morris et al. 2007). Asparagine level has been implicated in the formation of acrylamide during processing (Mottram et al. 2002). Evidently, the control of amino acid content in the tuber is important from several different quality perspectives.

7.2.4 Control of Potato Tuber Dormancy

Potato tuber apical buds exhibit the phenomenon of endodormancy: meristem growth is repressed under apparently favourable conditions for growth (Fernie and Willmitzer 2001). However, premature dormancy break can lead to deterioration in quality during potato tuber storage due to both disease-related and physiological

processes. Sprouting, following the loss of dormancy, is accompanied by changes that are detrimental to processing, including increases in reducing sugar content, respiration, water loss and glycoalkaloid content (Burton 1989). The length of the post-harvest dormancy period depends on both the genetic background of the cultivar and the prevailing environmental conditions during tuber development (Suttle 2004a). As it is often necessary to store potato tubers for periods beyond that of natural dormancy (generally 1–15 weeks), sprouting is controlled commercially by storage at low temperatures—expensive and affecting quality—and by the use of chemical sprout suppressants, such as chloropropham—expensive and leaving chemical residues in the food product (the European Union maximum residue level has recently been reduced to 10 ppm).

As with many plant developmental processes, roles for phytohormones in the control of dormancy have been investigated (reviewed in Wiltshire and Cobb 1996; Claassens and Vreugdenhil 2000; Fernie and Willmitzer 2001; Suttle 2004b). Although some effects of the phytohormones have been described, limited information exists on the mechanisms underpinning bud dormancy in potato and, especially, on the genetic and molecular processes involved. In particular, only limited forward and reverse genetic approaches have been applied to the investigation of the roles of the plant phytohormones, although such approaches are now technically feasible.

Several research avenues are currently being explored to enhance our understanding of the control of tuber dormancy. For example, it appears that the potato tuber life cycle is controlled by cycles of meristem activation and deactivation, mediated via symplastic association and disassociation of the tuber apical bud (Viola et al. 2001b). Thus, on dormancy release, the apical bud regains symplastic connection with the tuber and growth resumes (Viola et al. 2001b). Subsequent work examining dormancy release in buds of the mature tuber identified molecular markers in potato tuber buds that were induced or repressed specifically on release from endodormancy, in some cases prior to any visible sign of growth (Faivre-Rampant et al. 2004 a, b).

Other studies have demonstrated that potato tuber sprouting can be controlled by manipulation of carbohydrate metabolism (Geigenberger et al. 1998; Sonnewald 2001; Hajirezaei et al. 2003). Tuber sprout growth is initially supported by energy captured from sucrose breakdown. As inorganic pyrophosphate is a necessary cofactor for sucrose breakdown, removal of pyrophosphate by expression of a bacterial pyrophosphatase in transgenic tubers increases sucrose content and prevents its use as an energy supply. Consequently, sprout growth is inhibited significantly when sucrose is limited but accelerated when sucrose supply is increased. Transgenic approaches have also started to address the role of the plastid-derived isoprenoid hormones (particularly, cytokinins, GAs and abscisic acid) in the control of potato tuber dormancy. For example, over-expression of the gene encoding the first step of the isoprenoid biosynthetic pathway (catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase) leads to a much clearer separation of dormancy release and sprouting, enabling the initiating events in dormancy release to be temporally isolated from subsequent sprout growth (Morris et al. 2006). As for tuber initiation,

the use of microarray analyses, transgenic and genetic approaches, will enable the identification of the key regulatory genes in the coming years.

The control of dormancy in yam and Jerusalem artichoke is poorly understood. In yam, it would be beneficial to manipulate dormancy to allow off-season planting and, hence, obtain more than one generation per year. Recent work has defined three different phases of dormancy in yam and may provide a framework within which dormancy can be more effectively studied (Ile et al. 2006).

7.3 Summary

The importance of tubers as a nutrition source to humankind is clear. Our understanding of tuber development in potato has increased rapidly in recent years and will continue to do so as functional genomics and cell biology give us new insights into plant development. Translation of these advances to developing higher yielding crops of better nutritional quality is essential as the demands of population growth and environmental change increase on agriculture. Our knowledge of tuber development in other crops lags behind that of potato and the challenge remains to transfer knowledge from model systems, such as potato, to other tuber-bearing species such as yams.

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Chapter 8
Senescence

C. Zhou and S. Gan

8.1 Introduction

Like many other organisms, plants exhibit various life history patterns and possess a broad spectrum of longevity, ranging from a few weeks to several hundred years. Senescence is a universal phenomenon in all living organisms, and has been studied on yeast, animals, and plants. In plants, senescence is an important stage of development, and ultimately leads to death of a particular organ or whole plant. It is usually viewed as an internally programmed process that occurs in many different tissues, and serves different purposes.

Leaf senescence is a type of postmitotic senescence. In higher plants, it appears to be a form of programmed cell death (PCD) that can be regulated by an array of endogenous factors and environmental cues. This complex process involves orderly, sequential changes in cellular physiology, biochemistry, and gene expression. Although much research concerning the morphological, physiological, and biochemical changes associated with senescence has been performed, the controlling mechanisms of life span remain elusive. For the past decade, molecular genetic analyses of plant senescence, especially in the model plant *Arabidopsis thaliana*, have shed some light on this fundamental biological question. Previous reviews summarize the understanding of leaf senescence (Guo and Gan 2005; Lim and Nam 2005; Gan 2007; Lim et al. 2007). This chapter presents recent progress in various research areas of plant senescence, such as physiology, molecular biology, genomics, and biotechnology.

C. Zhou and S. Gan
134A Plant Science, Department of Horticulture, Cornell University, Ithaca, NY 14853-5904, USA
e-mail: sg288@cornell.edu

C. Zhou
College of Life Sciences, Hebei Normal University, Shijiazhuang, Hebei 050016, China

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8.2 Senescence in Plants

The life history of a cell consists of mitotic and postmitotic processes. A cell may undergo a certain number of divisions to produce daughter cells. After a limited number of divisions, the cell can no longer divide mitotically. Once a cell ceases mitotic division permanently, it is called mitotic senescence (Gan 2003). In the literature concerning yeast, germline cells, and mammalian cells in culture, this type of senescence is often referred to as cellular senescence, replicative senescence, proliferative senescence, or replicative aging (Sedivy 1998; Patil et al. 2005). In contrast, postmitotic senescence is the active degenerative process, leading to the death of a cell that no longer undergoes mitotic division. If a cell stops mitosis temporarily due to unfavorable conditions, but retains its mitotic capacity and can re-enter mitotic cycles to produce more daughter cells, then the temporarily undividing or resting status or process is called cell quiescence (Stuart and Brown 2006).

Plants exhibit both mitotic, postmitotic senescence, and cell quiescence (Gan 2003, 2007). Unlike replicative senescence in yeast and human cells in culture, mitotic senescence in plants is not controlled by telomere shortening. An example of mitotic senescence in plants is the arrest of apical meristems. A meristem consists of nondifferentiated germline-like cells that can divide many times to produce daughter cells. The latter can differentiate and form new organs, such as leaves and flowers. In the literature, the arrest of cells in the apical meristem is also called proliferative senescence (Hensel et al. 1994). Another example of mitotic senescence is the arrest of mitotic cell division at the early stages of fruit development. Fruit size is a function of cell number, cell size, and intercellular space, while cell number is the major factor. Cell number is determined at the very early stage of fruit development, and remains unchanged thereafter.

Postmitotic senescence, an active degenerative process, occurs in some plant organs, such as leaves and petals. Once the organs are formed, cells in these organs rarely undergo cell division. Their growth is contributed mainly by cell expansion; thus, their senescence, unlike mitotic senescence, is not due to an inability to divide. This type of senescence, involving predominantly somatic tissues, is similar to that of animal model systems such as *Drosophila* and *Caenorhabditis elegans*: with the exception of the germline, their adult bodies are postmitotic (Gan 2003).

Cell quiescence also occurs in plants. Cells of an apical meristem may stop dividing under unfavorable growth conditions. For example, the apical meristems of several trees may stop proliferation when they perceive short-day photoperiod signals, as short day indicates the arrival of the winter season. These meristematic cells retain their division capability during winter, and can resume division activity in spring.

8.3 Symptoms of Senescence

Obvious visual symptoms of leaf senescence are loss of chlorophyll, desiccation, and eventual death. At the cellular level, the senescence program unfolds in an orderly manner. Chloroplasts, which contain up to 70% of the proteins in a leaf cell,

are one of the first organelles to be targeted for breakdown. Other organelles, such as the peroxisome, also undergo biochemical changes as senescence proceeds. The nucleus, which is needed for gene transcription, and the mitochondria, which are essential for providing energy, remain intact until the last stages of senescence (Inada et al. 1998). Also associated with leaf senescence is the decline in the structural and functional integrity of cellular membranes (Thompson et al. 1998). During senescence, nutrients such as nitrogen, phosphorus, and sugars, released from the degradation of macromolecules in leaf cells, are reallocated to growing organs or storage tissues (Quirino et al. 2000).

8.3.1 Chlorophyll Degradation

Chlorophyll degradation is an integral part of the senescence syndrome, characterized by physiological and biochemical changes that aim at the recycling of nutrients from senescing tissues, such as leaves and fruits. Most reactions of chlorophyll degradation are known, and genes for some of the catabolic enzymes have been cloned recently (Hortensteiner 2006). The cleavage of the tetrapyrrole ring to produce red chlorophyll catabolite (RCC) by pheophorbide *a* oxygenase (PAO) is the key step for chlorophyll catabolism (Hortensteiner et al. 1998), which is often referred to as the PAO pathway. PAO is an Fe-dependent monooxygenase located at the envelope membrane of gerontoplasts. Electrons required to drive the redox cycle of PAO are supplied from reduced ferredoxin. Comparison of PAO activity with its mRNA and protein abundance during senescence of *Arabidopsis* indicated that *PAO* expression is regulated exclusively at the transcriptional level (Pruzinska et al. 2005). The expression of five *PAO* genes in *Arabidopsis* was shown to be up-regulated during dark-induced leaf senescence (Lin and Wu 2004). Microarray analysis (Zimmermann et al. 2004) indicates that *PAO* is also up-regulated under various stress conditions, such as osmotic stress and wounding. Therefore, the PAO pathway is activated not only during senescence, but also under other conditions that cause chlorophyll degradation.

8.3.2 Membrane Degradation

The symptoms of leaf senescence also include loss of membrane structural and functional integrity. Whether this occurs naturally, or is induced by environmental stress is evident from permeability analyses showing increased leakage of solutes when leaves undergo senescing. It is now accepted that membrane degradation is the result of enhanced catabolism of membrane lipids (Thompson et al. 1998). Lipid-degrading enzymes, such as phospholipase D, phosphatidic acid phosphatase, lytic acyl hydrolase, lipoxygenase, α-galactosidase, β-galactosidase, and galactolipase, appear to be involved in this process (Thompson et al. 1998). For example,

chloroplast thylakoid lipids are degraded initially by galactolipase and lipolytic acyl hydrolase (Woolhouse et al. 1984), and provide abundant carbon that can be mobilized and used as an energy source during senescence (Ryu and Wang 1995). A rice gene has been investigated encoding alkaline α-galactosidase, *Osh69*. The Osh69 protein is localized specifically in the chloroplast of senescing leaves, and is capable of hydrolyzing galactolipids, the major component of thylakoid membranes (Lee et al. 2004). The *Arabidopsis SAG101*, which encodes an acyl hydrolase, is induced at the early stages of leaf senescence, and its expression increases with the progression of leaf senescence (He and Gan 2002). Antisense suppression of *SAG101* retards the progression of leaf senescence, whereas inducible overexpression of the gene promotes precocious senescence in young leaves (He and Gan 2002).

8.3.3 Protein Degradation

Plant cells lose approximately two thirds of their soluble proteins during senescence (Inada et al. 1998). Up to 70% of leaf proteins are located in the chloroplasts. As supported by global gene expression analyses, chloroplast-localized protein-degrading enzymes, such as Clp protease (Lin and Wu 2004; Guo et al. 2004) and FtsH protease families (Andersson et al. 2004), could be involved in protein degradation in chloroplasts. *ERD1* (Nakashima et al. 1997) and several other Clp family genes (Nakabayashi et al. 1999) were isolated previously as senescence-associated genes (*SAGs*).

Several reports indicate that rubisco in senescing leaves could be degraded by vacuolar proteases (Yoshida and Minamikawa 1996; Minamikawa et al. 2001). During leaf senescence, substantial up-regulation of vacuolar cysteine proteases has been well documented (Buchanan-Wollaston et al. 2003) and supported by global transcriptome analyses (Bhalerao et al. 2003; Gepstein et al. 2003; Guo et al. 2004). Vacuolar proteases may play an important role in chloroplast protein degradation, at least at the final lytic stages after membranes are disrupted. At earlier stages of leaf senescence, when chloroplast membranes are intact, chloroplast protein degradation by vacuolar proteases may take place through the association of chloroplasts with the central vacuole, an aspect supported by electron microscopic studies on senescing leaves of French bean (Minamikawa et al. 2001).

Proteins in the cytoplasm are likely degraded through the ubiquitin pathway. Expression of a large portion of genes in the ubiquitin-26S proteasome pathway is associated with leaf senescence (Gepstein et al. 2003; Lin and Wu 2004; Guo et al. 2004). Mutation of ORE9, an F-box protein that interacts with the plant SCF complex (component of the ubiquitin E3 ligase complex), causes a delay in leaf senescence of *Arabidopsis* (Woo et al. 2001). The ubiquitin-mediated degradation of specific proteins appears to play an important role in the control of senescence. It has been postulated that *ORE9* may play regulatory roles in leaf senescence, by degrading proteins that negatively regulate leaf senescence.

8.3.4 Degradation of Nucleic Acids

A rapid decrease in nucleic acids occurs during leaf senescence. Total RNA concentrations are rapidly reduced with the progression of senescence. An initial decrease in RNA concentrations is observed for the chloroplast rRNAs and cytoplasmic rRNAs. The amounts of rRNA species may be regulated coordinately. A decrease in rRNA is followed by a decrease in cytoplasmic mRNA and tRNA. Although RNA concentrations decrease with an increase in the activity of several RNases, how each RNase functions exactly during senescence is not clear. Chloroplast DNA is likely the first DNA to be degraded along with chloroplast degeneration. Nuclear and mitochondrial DNAs are degraded at a later stage of senescence. Concomitantly, there is an increase in several DNase activities. Interestingly, there is a similarity between the meiotic senescence of animal cells and the mitotic senescence of plant cells, in terms of nuclear DNA metabolism. This appears to be involved in telomerases and chromosome fragmentation in mitotic senescence of plant cells, although these observations need to be substantiated with data from further studies.

8.3.5 Nutrient Remobilization

During leaf senescence, nutrients are mobilized from senescing leaves to actively growing regions, such as young leaves, floral buds, and developing fruits and seeds. The main transport route is the phloem. In *Arabidopsis*, concentrations of some macronutrients, including K, N, P, S, and C, and some micronutrients, such as Cu, Fe, Mo, Cr, and Zn, decrease by more than 40% during leaf senescence, but the nutrient remobilized to the greatest extent is N (90%; Himelblau and Amasino 2001). The concentrations of Mg, Na, and Ni were slightly less in the senescing leaves, while there was no difference between Ca, Co, and Mn in *Arabidopsis* leaves before and during senescence (Himelblau and Amasino 2001).

8.4 Regulation of Leaf Senescence

The onset and progress of leaf senescence are controlled by a number of external and internal factors. Internal factors that influence senescence include age, concentrations of plant growth regulators, and developmental processes, such as reproductive growth. Many environmental stressors and biological threats, such as extreme temperature, drought, nutrient deficiency, insufficient light/shadow/darkness, and pathogen infection, may induce senescence (Gan 2003). Regulatory factors that control the complex network of senescence are summarized below.

8.4.1 Age

In a natural setting, a plant inevitably encounters adverse and stressful environments that often induce leaf senescence. In the absence of external stresses, leaf senescence may occur in an age-dependent manner in many species (Hensel et al. 1993; Nooden and Penney 2001). This is particularly true in *Arabidopsis*. Individual leaves from wild-type *Arabidopsis* plants and various mutants in which the reproductive growth is either delayed (late-flowering mutants) or impaired (male or female sterile mutants) have an identical longevity. How age initiates leaf senescence is not well understood. It has been speculated that a decline in photosynthesis with age is a possible mechanism (Hensel et al. 1993). However, several lines of evidence indicate an antagonistic role of a decline in photosynthetic capability in determining leaf senescence. The *Arabidopsis* mutant *ore4* (which contains a lesion in a plastid ribosomal small subunit protein) displays reduced photosynthetic activity and delayed, rather than accelerated, age-dependent leaf senescence (Woo et al. 2002). Expression of the *Arabidopsis* gene *SAG12* (which encodes a cysteine protease) occurs specifically during senescence, and appears to be regulated by developmental age, and not by other endogenous or environmental factors (Noh and Amasino 1999). Analysis of the regulatory mechanism that controls *SAG12* expression might provide insight into the age-dependent mechanisms of leaf senescence.

8.4.2 Sugars

Sugars are known to act as signaling molecules at various stages of plant development, and for diverse physiological processes. Leaves are the primary site of sugar production by photosynthesis. Photosynthetic activity declines sharply during leaf senescence. This activity is also low in leaves grown in shade or complete darkness, which induces leaf senescence. It is therefore possible that a low concentration of sugars may induce leaf senescence (Hensel et al. 1993; Quirino et al. 2000). However, it has been suggested that a high sugar concentration may trigger the leaf senescence program, based on several reasons. Firstly, sugar concentrations are greater in senescing than in nonsenescing leaves of *Arabidopsis* and tobacco (Masclaux et al. 2000). Secondly, when yeast invertase (an enzyme that catalyzes the breakdown of sucrose into fructose and glucose) is expressed in the intercellular spaces of leaves from *Arabidopsis*, tobacco, and tomato, sugars accumulate and the leaves undergo premature senescence (Ding et al. 1993). Thirdly, tomato overexpressing hexokinase (HXK), which is believed to serve as a sugar sensor (Rolland et al. 2002), showed increased sensitivity to sugars. Moreover, leaves of these plants became precociously senescent. In contrast, the *Arabidopsis hxk1* knockout/null mutant plants displayed a delay in leaf senescence phenotype, even in the presence of high concentrations of sugars (Moore et al. 2003). Because sugars can serve as signal molecules and an important energy resource, it is possible that

control of senescence by sugar signaling is also affected by other factors, indicating that the mechanism in triggering leaf senescence may be complex. Various types or concentrations of sugars may possess different impacts on signaling pathways and/or sink-source relations, both of which are the important modulating factors of leaf senescence.

8.4.3 Reproductive Growth

Reproductive development may trigger leaf senescence in many plant species, especially in monocarpic plants. Monocarpic plants have single reproductive growth in their life history. Removal of flowers or fruits extends leaf longevity in many monocarpic plant species, such as soybean, pea, rice, and sunflower (Guo and Gan 2005). Leaf life span was extended by 50% in pea plants when flowers were removed (Pic et al. 2002). The removal of flowers delayed the onset of leaf senescence, and slowed down the senescence progression. However, not all experiments involving removal of flowers and/or fruits show a delay in senescence phenotype. Ear removal in maize plants can lead to either rapid or delayed leaf senescence, depending on the genotype. In *Arabidopsis*, leaf senescence appears to be unaffected by reproductive growth (Hensel et al. 1993; Nooden and Penney 2001).

8.4.4 Plant Growth Regulators

Leaf senescence can be induced or suppressed by various plant growth regulators (Gan and Amasino 1995; Gan 2007). Some plant growth regulators, such as ethylene, jasmonic acid (JA), ABA, and salicylic acid (SA), can promote leaf senescence, whereas other regulators, such as cytokinin, auxin, gibberellic acid (GA), and polyamines, may play important roles in the suppression of leaf senescence. Each regulator affects various developmental signal pathways.

8.4.4.1 Plant Growth Regulators That Induce Senescence

Ethylene

Ethylene plays an important role in plant growth and development, and it has long been seen as the key hormone in regulating the onset of leaf senescence. Symptoms of leaf senescence in many plant species can be either induced or retarded by application of either exogenous ethylene or its antagonists, respectively (Guo and Gan 2005). Leaf senescence is delayed in ethylene-insensitive mutants such as *etr1-1* (Grbic and Bleecker 1995) and *ein2/ora3* (Oh et al. 1997). However,

constitutive overproduction of ethylene in *Arabidopsis* and tomato plants did not cause precocious senescence, suggesting that ethylene alone is not sufficient to initiate leaf senescence (Grbic and Bleecker 1995). It has been postulated that age-dependent factors are required for ethylene-regulated leaf senescence. Transcriptional analyses of *Arabidopsis* hormone pathways during leaf senescence revealed that, of 69 genes involved in ethylene biosynthesis or signaling, 18 are up- or down-regulated (van der Graaff et al. 2006). This study also indicates a coordinated up-regulation of ethylene biosynthesis genes during leaf senescence in *Arabidopsis*, accompanied by changes in expression of several ethylene signaling components.

Jasmonic acid

Methyl jasmonate (MeJA) and its precursor jasmonic acid (JA) were first identified as bioactive substances that promote senescence in detached oat leaves (Ueda and Kato 1980). In *Arabidopsis*, it has been shown that JA levels are fourfold higher in senescing than in nonsenescing leaves, and genes encoding enzymes that catalyze most of the reactions of the JA biosynthetic pathway are differentially activated during leaf senescence (He et al. 2002). JA application induces premature senescence, and its concentration increases in senescing leaves. Several *Arabidopsis* mutants that are deficient in JA production or JA signal transduction do not exhibit a delayed leaf senescence phenotype (He et al. 2002). Transcriptional analyses revealed that 11 of 19 JA biosynthesis genes, and six of 11 JA signaling or response genes are up- or down-regulated, and some exhibit strikingly different regulation in leaf senescence (van der Graaff et al. 2006).

Salicylic acid

Salicylic acid (SA) plays a key role as mediator of plant stress responses, including disease and systemic acquired resistance. The levels of endogenous SA are fourfold higher in senescing than in nonsenescing leaves (Morris et al. 2000). It has been reported that treatment with SA up-regulates the expression of several *SAGs* (*NIT2*, *AtOSM34*, *SAG25*, *SAG26*, and *SAG29*; Quirino et al. 1999). This is consistent with a role for SA during later stages of the senescence program. Study of the *nahG*, *pad4*, and *npr1* mutants, which are defective in the SA signaling pathway, showed an altered expression pattern of a number of *SAGs*. Furthermore, a delay in yellowing, and reduced necrosis were observed in these plants (Morris et al. 2000). A transcriptome analysis in senescing *Arabidopsis* leaves from wild-type plants and SA-deficient *NahG* mutants revealed that many *SAGs* are dependent on the SA signaling pathway (Buchanan-Wollaston et al. 2005). Four of six genes that are involved in SA biosynthesis are regulated during natural leaf senescence (van der Graaff et al. 2006).

Abscisic acid

Abscisic acid (ABA) promotes senescence of detached leaves of various plant species, but it is less effective in leaves in planta (Weaver et al. 1998). The ABA concentration increases in senescing leaves, and exogenously applied ABA induces expression of several *SAGs* (Weaver et al. 1998), which is consistent with the effect on leaf senescence. Environmental stresses such as drought, high salt condition, and low temperature positively affect leaf senescence, and under these conditions ABA content increases in leaves. The ABA signaling and biosynthesis pathway is active during leaf senescence, and ABA can induce expression of several *SAGs* in *Arabidopsis*. ABA is considered an enhancer, rather than a triggering factor of leaf senescence. Four of 11 ABA biosynthesis genes are up-regulated, and of 30 ABA signaling genes, eight are up-regulated and only two are down-regulated during natural senescence (van der Graaff et al. 2006).

Brassinosteroids

Brassinosteroids (BRs) regulate the growth and differentiation of plants. External application of BRs induces senescence in mung bean leaves (He et al. 1996) and cucumber cotyledons (Zhao et al. 1990), and eBR induces a subset of potential *SAGs* in *Arabidopsis* (He and Gan 2001). Several *Arabidopsis* mutants that are deficient either in BR biosynthesis, such as *det2*, or in the BR signaling pathway, such as *bri1*, display a delayed leaf senescence phenotype (Clouse and Sasse 1998), and the *bri1* suppressor mutant exhibits an accelerated leaf senescence phenotype (Yin et al. 2002). However, transcriptome analysis suggested that BR biosynthesis does not significantly increase during senescence (van der Graaff et al. 2006). Consequently, it is necessary to perform more experiments to study the role of BRs in senescence.

8.4.4.2 Plant Growth Regulators That Suppress Senescence

Cytokinins

Cytokinins regulate cell division, as well as various metabolic and developmental processes, including senescence. Exogenous application of cytokinins (e.g., zeatin and benzyladenine) or their analogs delays leaf senescence, and even causes re-greening of yellowing leaves in a range of plant species such as soybean, tobacco, and *Arabidopsis* (Gan and Amasino 1996). In tobacco and many other plant species, concentrations of cytokinins in leaves decrease with the progression of senescence. In a transgenic study, the leaf senescence-specific promoter of *SAG12* in *Arabidopsis* was used to direct the expression of isopentenyl transferase (IPT). Plants harboring this system display a significantly delayed leaf senescence phenotype in many species (see Sect. 8.6). Overexpression of components of the cytokinin signal transduction pathway also delays leaf senescence in *Arabidopsis*

(Hwang and Sheen 2001), which further confirms the inhibitory role of cytokinins in leaf senescence.

Auxin

The role of auxin in leaf senescence is much less understood than that of ethylene, JA, or cytokinins, because it involves various aspects of plant development. However, auxin does play a role in leaf senescence. For soybean it has been shown that senescence can be retarded by application of auxin, and auxin treatment leads to a transient decrease in *SAG12* expression (Noh and Amasino 1999). The auxin concentration increases during leaf senescence. For example, in *Arabidopsis* senescing leaves, the IAA concentration is twofold greater than in nonsenescing leaves (Quirino et al. 1999). Consequently, IAA biosynthesis genes encoding tryptophan synthase (*TSA1*), IAA1d oxidase (*AO1*), and nitrilases (*NIT1-3*) are up-regulated during leaf senescence (van der Graaff et al. 2006).

Gibberellic acid

Gibberellic acid (GA) can induce seed germination, and modulate flowering time and the development of flowers, fruits, and seeds. In pea, exogenous GA3 can delay apical senescence, and endogenous GA concentration is lower in senescing than in flowering shoots (Zhu and Davies 1997). In detached *Arabidopsis* leaves, cytokinins, and to a lesser extent GA, delay chlorophyll degradation. Transcriptome analysis suggests that, during leaf senescence, some gibberellins are deactivated (van der Graaff et al. 2006).

External Factors

Leaf senescence can be induced by a number of different external factors, such as low/high light, extreme temperature, drought, flooding, ozone, nutrient deficiency, pathogen infection, wounding, and shading (Gan and Amasino 1997). Expression of *SAGs* can be induced in leaves exposed to many types of stress, such as darkness (Lin and Wu 2004), drought (Weaver et al. 1998; Pic et al. 2002), pathogen infection (Pontier et al. 1999), ozone treatment (Miller et al. 1999), UV-B exposure (John et al. 2001), and oxidative stress (Navabpour et al. 2003).

8.5 Molecular Genetic Regulation of Leaf Senescence

8.5.1 Gene Expression During Leaf Senescence

Leaf senescence is under direct nuclear control, and involves dramatic alteration in gene expression. A transcriptome analysis performed with a senescing leaf of *Arabidopsis* revealed that a large fraction of gene expression in a green leaf is

down-regulated, while there is also an up-regulation of the expression of up to 2,500 genes (Guo et al. 2004). In general, the down-regulated genes are involved in anabolic activities. Up-regulated genes, generally referred to as senescence-associated genes (*SAGs*), are mostly involved in catabolic activities. Microarray analyses of *Arabidopsis* cDNAs revealed that approximately 20% of the studied genes change their expression during leaf senescence (Buchanan-Wollaston et al. 2003). As discussed above, differential gene expression in leaf senescence could be triggered by many external and internal factors. The expression of thousands of *SAGs* leads to the execution of senescence, including the degradation of various macromolecules and remobilization of different nutrients. Different signals often induce different sets of genes (Weaver et al. 1998; He and Gan 2001), which in turn may initiate different biochemical/physiological processes. It has been postulated that multiple pathways are interconnected to form a regulatory network that controls leaf senescence (Gan and Amasino 1997). A simplified version of the network has been revealed by using *Arabidopsis* leaf senescence enhancer trap lines (He and Gan 2001).

8.5.2 Identification of SAGs

SAGs expression is required for senescence, because inhibitors of both transcription and translation prevent leaves from senescing. During the past decade, much effort has been made to isolate *SAGs*, and hundreds of *SAGs* have been cloned from various plant species by different approaches (Buchanan-Wollaston et al. 2003; Gepstein et al. 2003; Guo et al. 2004; Gan 2007). For example, a large-scale identification of *SAGs* via suppression subtractive hybridization has added 70 new members to the current SAG collection in *Arabidopsis* (Gepstein et al. 2003), the transcriptome associated with leaf senescence was examined by a large-scale EST analysis (Guo et al. 2004), a DNA microarray with 13,490 aspen ESTs was used to analyze the leaf transcriptome of aspen leaves during autumn senescence (Andersson et al. 2004), and the enhancer trap approach was used to identify *SAGs* and their functions (He et al. 2001). The *SAGs* identified by these studies include genes for potential regulatory factors, as well as genes executing the senescence program. The spectrum of *SAGs* is mostly consistent with known biochemical and physiological symptoms, and it also provides many new insights into the molecular events and their regulation during leaf senescence. Based on predicted physiological functions, current identified *SAGs* can be classified into several functional categories.

8.5.2.1 Transporters

A key role of senescence in plant tissues is the ordered degradation of macromolecules and mobilization of the products, during which transporters (TPs) are critically involved. In a large-scale microarray study, 74 putative TPs up-regulated

during developmental senescence were identified (Buchanan-Wollaston et al. 2005). During natural leaf senescence, 153 TPs are up-regulated (van der Graaff et al. 2006). The up-regulation for amino acid and oligopeptide TPs correlates with the extensive protein degradation taking place during senescence, and the subsequent need to export the breakdown products to the sink organs (Hortensteiner and Feller 2002).

8.5.2.2 Kinases and Receptor-Like Kinases

Senescence is associated with the induction of various genes that are potentially involved in signal and transduction, including protein kinases. For example, receptor kinases may trigger the transduction cascade of senescence signals via protein phosphorylation. Genes encoding receptor-like kinase (RLK), such as *SARK* in tomato (Hajouj et al. 2000), At5g48380 (Gepstein et al. 2003) in *Arabidopsis*, and *Paul27* in *Populus tremula* (Bhalerao et al. 2003), are induced by leaf senescence. The *Arabidopsis* genome has a large gene family of *RLKs* consisting of more than 610 genes. Transcripts of 44 *RLK* genes are found in senescent leaves (Guo et al. 2004). The mitogen-activated protein kinase (MAPK) signal cascades are also involved in leaf senescence. *Arabidopsis* genes encoding for three MAPKs, three MAPKKs, nine MAPKKKs, and one MAPKKKK are represented in the aforementioned senescent leaf EST database (Guo et al. 2004). AtMKK9 regulates leaf senescence through phosphorylation of AtMPK6 (Zhou et al. 2009).

8.5.2.3 Transcription Factors

There are approximately 1,500 transcription factors (TFs) in the *Arabidopsis* genome that belong to more than 30 gene families based on their DNA-binding domains, and more than 130 of these are represented in the *Arabidopsis* leaf senescence EST collection (Guo et al. 2004). These senescence TFs are in the families of *WRKY*, *NAC*, *AP2/EREBP*, *C2H2*, *C3H*, *MYB*, *bZIP*, and *bHLH*, amongst others. *WRKYs* are perhaps the best studied among the leaf senescence-associated TFs. *WRKY6* (Robatzek and Somssich 2001) and *SIRK* (Robatzek and Somssich 2002) are expressed during leaf senescence in *Arabidopsis*. *SIRK* encodes a receptor kinase. It is likely that *WRKY6* binds to the promoter region of SIRK to regulate the expression of this gene. Another *WRKY* gene, *WRKY53*, was shown to be induced at an early stage of leaf senescence (Hinderhofer and Zentgraf 2001). *Arabidopsis* plants overexpressing this gene displayed an accelerated senescence phenotype. In contrast, when the gene was suppressed by using RNAi or insertional mutation, the onset of leaf senescence was delayed (Miao et al. 2004). NAC proteins make one of the largest families of plant-specific TFs (Guo et al. 2004). Several members of the NAC domain proteins showed enhanced expression during senescence (John et al. 1997). *AtNAP*, an *Arabidopsis* NAC family TF, has been shown to play an important role in regulating leaf senescence (Guo and Gan 2006).

The ancestral wild wheat allele encodes a NAC TF (*NAM*-B1) that accelerates senescence, and increases nutrient remobilization from leaves to developing grains. Reduction in RNA levels of the multiple *NAM* homologs by RNA interference delayed senescence by more than 3 weeks, and reduced wheat grain protein, zinc, and iron content by more than 30% (Uauy et al. 2006). The function of many other potential TFs, which have been identified as *SAGs* through DNA microarray analysis, remains to be elucidated.

8.5.2.4 Autophagy Genes

During senescence, different pathways contribute to the degradation of proteins and other macromolecules, one of these being autophagy (ATG). Involvement of the autophagy pathway during leaf senescence is indicated by an increase in the expression of the autophagy genes, such as *ATG7* and *ATG8* (Doelling et al. 2002). Autophagy is an intracellular process for vacuolar bulk degradation of cytoplasmic components, and is known to be required for nutrient recycling. As observed in yeast, autophagy may contribute to maintaining cell viability during senescence/starvation. Nineteen of the 21 *Arabidopsis ATG* genes are up-regulated during leaf senescence (van der Graaff et al. 2006). This suggests that most *ATG* genes are coordinately up-regulated at a stage in developmental senescence when chlorophyll is degraded.

8.6 Genetic Manipulation and Application of Leaf Senescence

Manipulation of leaf senescence is highly desirable in practice. If leaf senescence were to be inhibited, one could obtain increased crop yields and greater biomass accumulation, and extend the storage of some vegetable crops and their shelf-life. Conversely, promotion of leaf senescence is also needed. For example, cotton bolls are generally harvested mechanically; green leaves are easily damaged, and the leaf trash reduces fiber quality.

As discussed above, the initiation and progress of leaf senescence are controlled by many internal and external factors. So, manipulation of any of these factors can affect senescence. Surgical removal of the inflorescence can delay leaf senescence. Low temperature has been widely used to prolong the storage and shelf-life of many vegetables and fruits. Exogenous applications of cytokinins have been used to effectively delay senescence of vegetables. Antagonists of ethylene action, such as Ag^{+} and 1-MCP, are commonly used in postharvest storage to prevent plants from senescing (Blankenship and Dole 2003). The development of molecular biology and plant transformation technology makes it possible to manipulate senescence using genetic modification. For example, transgenic tomato plants with suppressed expression of two genes encoding for the ethylene biosynthetic

enzymes, ACC synthase (Oeller et al. 1991) and ACC oxidase (Aida et al. 1998), showed significantly reduced ethylene production and retarded fruit senescence.

SAG12 was first isolated by differential screen of an *Arabidopsis* leaf senescence cDNA library by Gan (1995). It encodes an apparent cysteine proteinase, and its expression is highly senescence-specific (Lohman et al. 1994; Gan 1995). IPT is an enzyme that catalyzes the first committed and rate-limiting step of cytokinin biosynthesis, condensation of dimethylallyl pyrophosphate (DMAPP) and 5′AMP to isopentenyladenosine (IPA) 5′-phosphate (Gan and Amasino 1996). The *SAG12* promoter was fused to *IPT* to form an autoregulatory cytokinin production system (Gan and Amasino 1995). At the onset of leaf senescence, the senescence-specific promoter activates the expression of *IPT*, resulting in an increase in the cytokinin concentrations; in turn, this prevents the leaf from senescing. The inhibition of leaf senescence will render the senescence-specific promoter inactive to prevent cytokinins from accumulating to very high levels; overproduction of cytokinins may interfere with other aspects of plant development. Because cytokinin production is targeted to senescing leaves, overproduction of cytokinins before senescence will be avoided. Leaf senescence in transgenic tobacco plants containing this autoregulatory cytokinin production system was efficiently retarded, without any other developmental abnormalities (Gan and Amasino 1995). In the past decade, this autoregulatory senescence inhibition system has been applied in many plants, including some important agronomic and horticultural crops such as rice, rape, tomato, cassava, broccoli, lettuce, cauliflower, bok choy, petunia, alfalfa (Guo and Gan 2007), and wheat (Sykorova et al. 2008). Significant delay of leaf senescence is the most striking phenotype of transgenic plants harboring the SAG-*IPT* chimeric gene. Not only leaf senescence was delayed, but also yield and biomass production were increased in SAG12-*IPT* rice, while resistance to drought stress was increased in SAG12-*IPT* tobacco.

As discussed above, significant progress has been made in deciphering the physiological, cellular, biochemical, and molecular mechanisms underlying leaf senescence, which makes it possible to design other strategies to inhibit or to promote leaf senescence.

8.7 Conclusions and Outlooks

Senescence is an integral part of plant development. Like many other developmental processes, it is a genetically controlled program regulated by a range of environmental and autonomous factors. Recent progress has been made in the molecular biology of leaf senescence research. Approximately 2,500 genes that are expressed in senescing leaves of *Arabidopsis* have been identified, and a few of the genes have been characterized functionally. Current studies have revealed that the regulation of leaf senescence is complex, and likely involves a complicated network. These studies have laid a foundation for unraveling the molecular regulatory mechanisms underlying leaf senescence. In order to fully understand leaf

senescence, it is necessary to continue characterizing the many *SAG*s, especially those encoding transcription factors and those encoding components of signal transduction pathways, by using various functional genomics approaches.

The ultimate goal of studying leaf senescence is rooted in the regulatory mechanisms of senescence, to design ways to manipulate this process for agricultural improvement. The current molecular genetic approaches that have been used in delaying senescence are based on plant hormone biology, by either blocking ethylene production or enhancing cytokinin production. As discussed in this chapter, some important regulators of leaf senescence have been identified, so that one could expect that new strategies involving altering expression of these key factors, individually or in combination, will be developed and applied to manipulate leaf senescence.

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Part III
Reproductive Growth and Development

Chapter 9
Floral Organ Initiation and Development

M. Bemer and G.C. Angenent

9.1 Introduction: the Angiosperm Flower

Flowering plants (angiosperms) form the largest group of terrestrial plants, with more than 250,000 species. They appeared rather suddenly in the fossil record during the Jurassic (208–145 million years ago), but diversified tremendously in the relatively short time of their existence. The ecological dominance of the angiosperms over the gymnosperms, ferns and mosses is the result of three unique beneficial features, namely (1) the evolution of the carpel, (2) the emergence of double fertilization and (3) the appearance of the flower. The modern angiosperms did not appear until the Early Cretaceous (145–125 million years ago), when the final combination of these three characteristics occurred (Maere et al. 2005).

Angiosperms evolved from gymnosperms, which bear their seeds ‘naked’ in strobili (cones). Although the gymnosperm seed cones are optimized for seed dispersal, specialized structures that stimulate pollination are generally not present. Gymnosperms have separate male and female reproductive structures, and pollen is dispersed by the wind. Angiosperms evolved a bisexual flower, which often consists of four whorls. The inner or fourth whorl contains the female reproductive structure, the carpel, which encloses the ovules. The stamens, representing the male reproductive organs, contain the pollen and surround the carpel in the third whorl. The outermost two whorls give rise to the petals, which have an important function in attracting pollinators, and the sepals, which protect the immature flower. The sterile whorls of the plant are also indicated as perianth. Simultaneously with

M. Bemer and G.C. Angenent
Department of Plant Cell Biology, Radboud University Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands

G.C. Angenent
Plant Research International, Bioscience, Droevendaalsesteeg 1, 6708 PB Wageningen The Netherlands
e-mail: gerco.angenent@wur.nl

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the evolution of the flower, insects evolved to effectively pollinate the flowers and the success of fertilization increased considerably.

Angiosperm species show a tremendous diversity in both inflorescence architecture and floral shape. Often, the inflorescence and flower forms typify a plant family, but the same type of inflorescence architecture or flower can also be found in unrelated families, as a result of adaptive selection (Benlloch et al. 2007). Flowering plants can be further subdivided into three major classes, the basal angiosperms (4%), the monocotyledons (22%) and the eudicotyledons (74%). Eudicotyledons typically have the floral organs arranged in four whorls, have a fixed number of organs in each whorl (four or five or a multiple thereof) and have a perianth with distinct sepals and petals. In contrast, the two outer whorls of monocotyledons are often identical or rudimentary. The monocotyledon whorls usually give rise to three floral organs or a multiple thereof. The basal angiosperms are thought to be the earliest angiosperms, and exhibit a great diversity in floral form and structure (Erbar 2007). Numerous plant species show variations on this typical flower plan, for example, by developing lodicules instead of the perianth (grasses) or by producing mono-sexual flowers.

Several model species, representing different flower types, have been subjected to extensive research to elucidate the molecular background of inflorescence architecture and floral shape. Important model species in flower research are the eudicotyledon species *Arabidopsis thaliana* (Brassicaceae), *Antirrhinum majus* (Plantaginaceae) and *Petunia hybrida* (Solanaceae), for which various genetic and molecular tools are available. Monocotyledon model species are the crops *Oryza sativa* (rice, Poaceae) and *Zea mays* (maize, Poaceae).

Despite the high diversity in inflorescence architecture and flower morphology, the genetic networks controlling the development of both structures are largely conserved among flowering plants. This chapter reviews the current knowledge on the molecular processes involved in floral organ initiation and development in angiosperms by means of the key model species *Arabidopsis*. Subsequently, the data from *Arabidopsis* are compared with available data from other model species, to illustrate conservation or divergence in the evolution of the flower.

9.2 The MADS Box Family of Transcription Factors

There is substantial evidence that two large-scale genome duplications occurring at the beginning of angiosperm evolution provided a source of genetic material for the evolution of angiosperm features (Becker et al. 2000; Maere et al. 2005). Both duplication events resulted in a remarkable increase in the number of MADS box transcription factors in angiosperms. Whereas animals and fungi contain only a few genes belonging to this family, the genomes of angiosperms contain numerous MADS box genes (Alvarez-Buylla et al. 2000b). Research revealed that many duplicated MADS box genes have been recruited as homeotic genes for the development of two of the three unique angiosperm features, the carpel and the

flower. MADS box transcription factors are named after the first four characterized genes, namely *MCM1* (yeast), *AGAMOUS (AG; Arabidopsis), DEFICIENS (DEF; Antirrhinum*) and the mammalian SERUM RESPONSE FACTOR (*SRF*; Schwarz-Sommer et al. 1990), and all share a conserved N-terminal domain of approximately 60 amino acids. In plants, the family can be subdivided into type I genes and MIKC-type genes. MIKC-type proteins contain, in addition to the MADS box, a highly variable intervening (I) region, important for protein interaction selectivity, a conserved keratin-like K-domain region, involved in dimerization, and a C-terminus that supports the formation of higher-order protein complexes and may serve as transcription activator or suppressor domain (Krizek and Meyerowitz 1996; Riechmann et al. 1996a; Yang et al. 2003). Before the appearance of the angiosperms or early in angiosperm evolution, duplications in the MIKC-type subfamily resulted in several functionally divergent classes important for developmental processes, such as the determination of floral organ identities (Becker and Theissen 2003). Lineage-specific duplications within these classes gave rise to paralogous genes that often function in a redundant manner. An important feature of MADS box proteins is that they interact with each other in different combinations to form multimeric complexes (Gutierrez-Cortines and Davies 2000; de Folter et al. 2005), thus creating a large collection of different transcription regulatory complexes (Theissen and Saedler 2001) to control the expression of numerous downstream genes.

9.3 Change from Vegetative Growth to Reproductive Growth

9.3.1 Transition to the Reproductive Phase

The moment at which transition to flowering occurs in a plant is dependent both on the plant's developmental state and on environmental factors. In *Arabidopsis*, four pathways have been identified that regulate the transition to flowering. The long day and vernalization pathways respond to environmental signals, whereas the developmental state of the plant is monitored by the autonomous and gibberellic acid (GA)-dependent pathways (Moon et al. 2005). In all four pathways, genes are active that regulate flowering time by either promoting or inhibiting the expression of the flowering pathway integrators *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) or *LEAFY* (*LFY*; Moon et al. 2005). The three genes have both overlapping and independent functions in promoting the transition to flowering. *FT* and *SOC1* are both very important for the integration of the long day, vernalization and autonomous pathways, whereas only *SOC1* is responsive to the GA pathway (Lee et al. 2000; Onouchi et al. 2000; Samach et al. 2000). Although *LFY* is regulated by both the long day and the GA pathways (Blazquez and Weigel 2000), flowering time is only slightly delayed in *lfy* mutants. Studies of double and triple mutants of the three flowering pathway integrators showed that *FT* and *SOC1* have a more direct function in determining flowering

time, whereas the main function of *LFY* is in the initiation of flower formation (Moon et al. 2005). The upregulation of *FT*, *SOC1* and *LFY* promotes the conversion of the shoot apical meristem into the inflorescence meristem (IFM) and induces the expression of the floral meristem (FM) identity genes, which are responsible for specifying FM identity.

9.3.2 Induction of the Floral Meristem

Specification of the FM in *Arabidopsis* is promoted by the FM identity genes *LFY*, *UNUSUAL FLORAL ORGANS* (*UFO*), *APETALA 1* (*AP1*), *CAULIFLOWER* (*CAL*) and *FRUITFULL* (*FUL*; Schultz and Haughn 1991; Bowman et al. 1993; Wilkinson and Haughn 1995; Ferrandiz et al. 2000). However, like many angiosperm species, *Arabidopsis* does not have a single terminal flower, but produces numerous flowers from the IFM, resulting in a raceme inflorescence. To establish this architecture, the IFM needs to remain indeterminate, and expression of the FM identity genes has to be restricted to the laterally arising determinate FMs. Maintenance of IFM identity is guaranteed by the expression of *AG-LIKE 24* (*AGL24*) and *TERMINAL FLOWER 1* (*TFL1*), which repress the expression of the FM identity genes in the IFM (Shannon and Meeks-Wagner 1993; Yu et al. 2004). Reversibly, *LFY*, *AP1* and *CAL* inhibit the expression of *AGL24* and *TFL1* in the FM, and it has been demonstrated that the interplay between the expression of the IFM and FM identity genes specifies the boundaries of both meristems (Weigel et al. 1992; Liljegren et al. 1999; Ratcliffe et al. 1999).

The six floral meristem identity genes in Arabidopsis are not equally important for the specification of the FM. Only the *lfy* single mutant shows a dramatic conversion of flowers to shoots, especially in the most basal nodes, whereas single mutants of *ap1, ufo, cal* and *ful* have no or only a minimal effect on floral initiation (Irish and Sussex 1990; Schultz and Haughn 1991; Bowman et al. 1993; Wilkinson and Haughn 1995; Kempin et al. 1995; Ferrandiz et al. 2000). In response to the transition to flowering, the expression of *LFY* is induced rapidly in the initiating floral primordia (Simon et al. 1996). *LFY* directly upregulates the expression of the other FM identity genes *AP1, CAL* and possibly also *FUL*, three members of the MADS domain family (Ferrandiz et al. 2000; William et al. 2004). In turn, *AP1*, *CAL* and *FUL* further upregulate *LFY* expression, until the levels of *LFY* exceed a certain threshold required for the actual initiation of flowering. *Ap1 cal ful* mutant plants fail to produce any kind of flower structures, because the level of *LFY* expression never reaches the critical threshold (Blazquez et al. 1997; Ferrandiz et al. 2000). Together, the FM identity genes initiate a cascade of gene expression required for the specification of the floral organs (Fig. 9.1).

The two key regulators in the establishment of FM identity, *LFY* and *AP1*, have also been subjected to studies in various other plant species. *LFY* encodes a transcription factor that has been found only in the plant kingdom and does not belong to a multigene family (Benlloch et al. 2007). The gene is present in all land

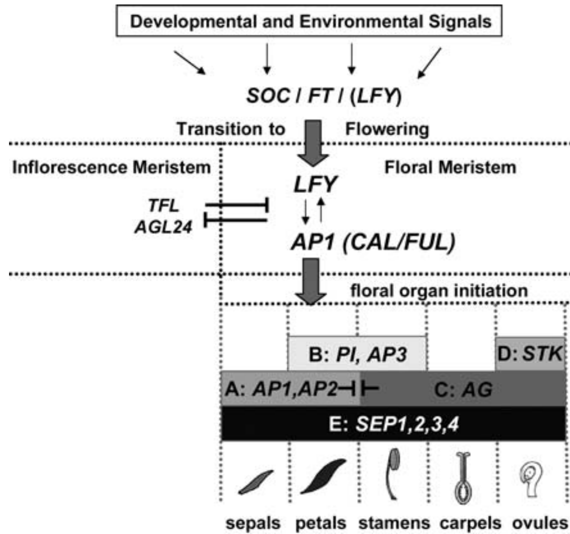

Fig. 9.1 Gene regulatory network involved in flower induction and floral organ formation in *Arabidopsis*. Only the key genes are indicated. The letters A, B, C, D and E represent the functions required for the identity specification of the different floral organs according to the ABCDE model (Ferrario et al. 2003)

plants analyzed to date, usually as a single copy. In contrast to *LFY*, orthologs of the MADS box gene *AP1* have been found only in angiosperm species. *AP1* shares a high sequence homology with the other two MADS box genes involved in FM identity, *CAL* and *FUL*. *FUL* and *AP1* belong to different gene clades, which originated at the base of the core eudicotyledons (Litt and Irish 2003). Because of the high homology between the two genes, it is often difficult to assign homologs from other species to one of the two clades. The redundancy of *AP1*/*CAL* has been documented only in the *Brassicaceae* and results from a recent gene duplication event in this family (Lawton-Rauh et al. 1999).

LFY and *AP1* homologs have been studied in species with indeterminate inflorescences, like *Arabidopsis*, and species with determinate inflorescences, such as *Petunia*. The two types of inflorescences differ in the location of the IFM and FM. Indeterminate inflorescences have an indefinitely growing apical meristem, which laterally produces FMs meristems, whereas in determinate inflorescences the main shoot terminates in a flower, while new IFMs are formed laterally immediately below the terminal flower (Benlloch et al. 2007). *LFY* homologs have been characterized in *Antirrhinum* (*FLORICAULA; FLO*), pea (*UNIFOLIATA; UNI*), tomato (*FALSIFLORA; FA*), petunia (*ABERRANT LEAF AND FLOWER; ALF*) and maize (*ZEA FLO*/*LFY 1,2; ZFL1*, *ZFL2*; Coen et al. 1990; Hofer et al. 1997; Souer et al. 1998; Molinero-Rosales et al. 1999; Bomblies et al. 2003). All these homologs are involved in the transition to flowering, and the mutants generally show a full conversion of the FM to an IFM. Yet the expression of the *LFY* homologs often shows particular features that are related to the architecture of the inflorescences. For example, the expression of *FA* and *ALF* in tomato and petunia respectively, has been reported also for the IFM, probably related to the formation of terminal flowers by their inflorescences (Benlloch et al. 2007).

Similarly, the characterized *AP1*-like genes of other species all play a role in establishing FM identity. In species outside the Brassicaceae, loss-of-function phenotypes of AP1-like genes often show a more severe phenotype with an almost complete replacement of FMs by IFMs, as has been reported for the *squamosa* mutant (*SQUA; Antirrhinum*), the *pim* mutant (*PROLIFERATING INFLORESCENCE MERISTEM*; pea) and the *mtpim* mutant (*MtPIM; Medicago truncatula*; Huijser et al. 1992; Taylor et al. 2002; Benlloch et al. 2006). The stronger inflorescence phenotype can probably be explained by the absence of the redundant *CAL* gene.

9.3.3 Initiation of Flower Primordia

The FM identity genes specify the FM, but do not determine the precise location where the flower primordia are formed. The spacing between leaf or flower primordia is regular and follows a distinct pattern. Often, the arrangement of the leaves or flowers around the stem, called phyllotaxis, occurs as a spiral, commonly with an angle of 137.5° (the golden angle; Kuhlemeier 2007). The mechanism underlying the regular arrangement has been puzzling scientists for centuries, but characterization of the *pin1* mutant, defective in polar auxin transport, revealed that phyllotaxis is controlled by the phytohormone auxin, i.e. indole-3-acetic acid. In *Arabidopsis*, the subcellular localization of the transmembrane PIN1 (PIN-FORMED 1) protein determines the direction of auxin transport in the stem. Inflorescences of the *pin1* mutant do not form any flowers, and the size, shape and position of the leaves are aberrant (Okada et al. 1991; Reinhardt et al. 2003; Cheng and Zhao 2007). This phenotype can partly be rescued by the application of exogenous auxin, which induces the outgrowth of primordia at the place of application. These data resulted in the following model, proposed by Reinhardt et al. (2003). Auxin accumulation induces a primordium, which will absorb auxin, resulting in the depletion of auxin in the surroundings of the primordia. A new auxin maximum can occur only at a certain minimal distance from the existing primordia, giving rise to a regular pattern of primordia formation. In addition to the regulation of phyllotaxis, auxin also plays essential roles in specifying the number and identity of floral organs (Cheng and Zhao 2007), as can be concluded from analysis of the *ettin* and *yucca* mutants (Sessions et al. 1997; Cheng et al. 2006).

9.3.4 Floral Organ Specification

If both the FMI genes, *LFY* and *AP1*, are induced and auxin accumulation occurs, flower primordia are initiated and the floral organ identity genes will be activated to give rise to the different floral organ primordia. The organ primordia form the sepals, petals, stamens and the carpels, which enclose the ovules, and emerge from

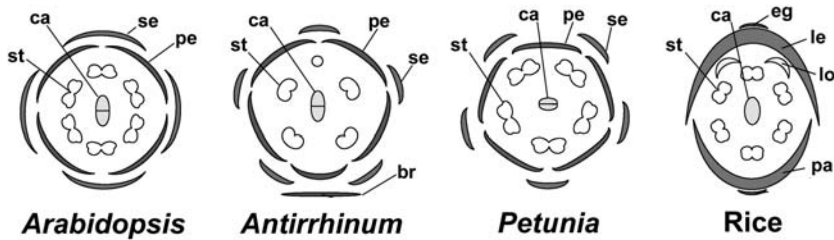

Fig. 9.2 Schematic representation of the arrangement of the floral organs in *Arabidopsis*, *Antirrhinum*, *Petunia* and rice. Abbreviations: se, sepals; pe, petals; st, stamens; ca, carpels; br, bracts; eg, empty glumes; lo, lodicules; pa, palea; le, lemma

the FM in concentric whorls (Fig. 9.2). Genetic research on the establishment of the floral organ identities was initially executed in *Arabidopsis* and *Antirrhinum* and resulted in the identification of several homeotic mutants, which were affected in the development of one or more of the floral organs (Bowman et al. 1989; Schwarz-Sommer et al. 1990). Although *Arabidopsis* and *Antirrhinum* are only distantly related, both species revealed to use homologous mechanisms to specify the identity of the floral organs. The analysis of the homeotic mutants from both species led to the formulation of the famous ABC model for flower development by Coen and Meyerowitz (1991). This model describes the combinatorial interaction of three classes of genes, A, B and C, in the formation of the floral organs. Additional research, also carried out in petunia, resulted in the discovery of two other functional classes, D and E, and the model was extended to the ABCDE model (Fig. 9.1; Angenent et al. 1995b; Colombo et al. 1997; Alvarez-Buylla et al. 2000a; Ditta et al. 2004).

The E-class genes have a broad expression pattern and are essential for the formation of all floral organs. The other functional classes have a more specific role in establishing the identity of the floral organs, in combination with the E-class genes. A+E-class genes define the sepals; A, B+E together determine petal development. B, C+E function is necessary for the formation of the stamens, while expression of C+E-class genes establishes carpel identity. D function is required for the formation of the ovules, in combination with the C and E functions (Theissen 2001). The model predicts the homeotic conversions that occur when one of the different gene classes is not functioning. If the A-class genes are eliminated, the C-class genes are also expressed in the first two whorls, and the four whorls of the flower give rise to carpels (instead of sepals), stamens (instead of petals), stamens and carpels (Jofuku et al. 1994). Loss of B-function genes results in a flower consisting of sepals, sepals, carpels and carpels (Schwarz-Sommer et al. 1990), while omission of C function leads to flowers with sepals, petals, petals and sepals (Yanofsky et al. 1990). Repression of the D function gives rise to flowers that bear carpel-like structures on the ovaries, instead of ovules (Colombo et al. 1997). The most dramatic phenotype is observed when the function of the E-class genes is

impaired, resulting in the conversion of all floral organs into leaf-like structures (Ditta et al. 2004).

Representatives of the A-, B-, C-, D- and E-class genes have been identified in all angiosperm species that have been studied so far (Erbar 2007). Classes may be represented by only one gene or by several genes, depending on the number of duplications that have occurred in the different angiosperm lineages. All genes that play a role in the ABCDE model belong to the subfamily of MIKC-type MADS box transcription factors, except for *APETALA2* (*AP2*), which fulfils the A function in some angiosperm species but belongs to the AP2-like gene family.

9.4 Floral Quartet Model

The ABCDE model describes the interaction of different classes of genes to establish the identity of the different floral organs. However, the model does not explain the mechanisms by which the homeotic genes and their gene products interact. To address this problem, Theissen and Saedler (2001) proposed the 'floral quartet model', which is based on the capacity of MADS domain proteins to interact with each other and form multimeric complexes (Fig. 9.3). The higher-order protein complexes bind to the *cis*-regulatory elements in the promoter regions of target genes to induce or repress their expression. The quartet model postulates that five different tetrameric complexes are formed, each specifying the identity of one of the floral organs. Theissen and Saedler (2001) hypothesize that the two dimers of each tetramer bind two different *cis*-elements in the regulatory region of a target gene, which causes the DNA to bend, and regulates the transcription of the gene. Although the 'floral quartet model' is still hypothetical, studies in yeast and living plant cells revealed that many of the MADS domain proteins indeed interact with proteins from the other classes according to the ABCDE model (Riechmann et al. 1996b; de Folter et al. 2005). Moreover, formation of several of the predicted higher-order complexes has been demonstrated (Honma and Goto 2001; Favaro et al. 2003; Ferrario et al. 2003).

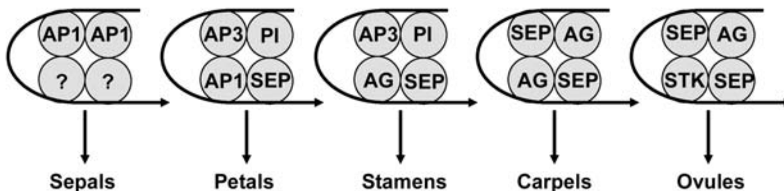

Fig. 9.3 The floral quartet model (based on Theissen and Saedler 2001; Favaro et al. 2003). Schematic representation of the putative quartet complexes that promote the formation of the floral organs in *Arabidopsis* by binding to the target DNA sequences

9.4.1 A Function

The role of the A-class genes in the formation of the sepals and petals can be separated into two different functions: a cadastral function in the repression of the C-class genes in the outer two whorls, and a homeotic function in the specification of the perianth organs. In contrast to the other functions of the ABCDE model, the A function appears to be differentially regulated in different model species, and the genes that fulfil the A function are not generally conserved among the angiosperms (Rijpkema et al. 2006; Cartolano et al. 2007).

The A function in *Arabidopsis* has been reported to be executed by two genes, *AP1*, a MADS box gene, and *AP2*, belonging to the AP2-like transcription factor family (Komaki et al. 1988; Irish and Sussex 1990). The *ap1* mutant does not produce petals, and forms bract-like leaves instead of sepals (Irish and Sussex 1990). However, petal formation in *ap1* mutants is largely restored by a mutant *agl24* allele, indicating that *AP1* is not directly involved in establishing the identity of the sepal and petal primordia, but rather plays a role in repressing *AGL24* in the first two whorls (Yu et al. 2004). In contrast to *AP1, AP2* exhibits a real A function by promoting the development of sepals and petals and by repressing the C function in the first and second whorl. In accordance with these functions, *ap2* mutants show conversion of sepals into carpelloid structures and of petals into stamenoid structures (Komaki et al. 1988; Bowman et al. 1991; Jofuku et al. 1994).

AP2-like genes have also been characterized in *Antirrhinum* and *Petunia*, but they revealed to play less important roles in the specification of the perianth organs. In *Antirrhinum*, the *AP2*-like genes *LIPLESS1 (LIP1)* and *LIP2* are redundantly involved in sepal and petal formation, but do not function in repressing the C function in the outer two whorls. A *lip1/lip2* double mutant produces bract-like organs in the first whorl and reduced petals without lip or palate in whorl two (Keck et al. 2003). Studies in *Petunia* revealed a close homolog of the *Arabidopsis AP2* gene, named *PhAP2A*, which exhibits an expression pattern during flower development similar to that of *AP2* in *Arabidopsis*. However, the analysis of several *phap2a* mutants did not reveal a phenotype in the perianth, indicating that the *Petunia AP2* homolog is not involved in floral organ specification or that redundant *AP2*-like genes fulfil this function together (Maes et al. 2001). Both in *Antirrhinum* and *Petunia*, mutants have been identified that do show ectopic C-gene expression in the outer whorls and exhibit conversion of petals to stamens in the second whorl. Since *AP2*-like genes are not involved in this aspect of the A function, other factors must be responsible for these mutant phenotypes. Using transposon tagging and map-based cloning strategies, Cartolano et al. (2007) cloned the *BLIND (BL)* and *FISTULATA (FIS)* genes of *Petunia* and *Antirrhinum* respectively, responsible for the ectopic C expression in the corresponding mutants. Both genes were found to encode homologous microRNAs (miRNAs), named miRBL and miRFIS, related to the large miR169 family. miRNAs control gene expression by recognizing short complementary sequences in their target transcripts, which are subsequently silenced by cleaving or translational inhibition. Detailed analysis of the *BL*

and *FIS* genes and their targets suggests that C-gene expression in the outer whorls of *Petunia* and *Antirrhinum* flowers is kept below a threshold level by inhibition of the direct target NF-YA, which is an activator of C-gene expression (Cartolano et al. 2007).

The A function has also been studied in a diversity of other plant species among the eudicotyledons, monocotyledons and basal angiosperms. However, although *AP1* and *AP2* homologs were identified in these species, none of these homologs appear to encode an A-function protein (Yu et al. 1999; Fornara et al. 2004; Litt 2007; Soltis et al. 2007a). Possibly, the A function is not conserved among angiosperms and non-homologous genes fulfil the function in different species. Alternatively, a discrete perianth identity function may not be required, and the existence of an A function may be specific for the Brassicaceae (Litt 2007). In this case, floral organ identity outside the Brassicaceae may be regulated according to a BCDE model. In this model, the presence of an FM, specified by *LFY* and *AP1* homologs, is sufficient to initiate sepals instead of leaves, while petal identity is specified by B+E and AP1. The repression of C function in the first two whorls is probably executed by cadastral genes, rather than by real floral organ identity genes. Whether this function is generally executed by miRNA control, like in *Petunia* and *Antirrhinum*, needs to be investigated. Future studies on the genes involved in sepal and petal formation in species outside the Brassicaceae are required to elucidate the status of the A function.

9.4.2 B Function

B-function genes fulfil a homeotic function in the determination of petal and stamen identity in the eudicots. They belong to the oldest lineages of plant MADS box genes and have been found also in gymnosperms. Despite their early origin, B-class genes are considered to be very important for angiosperm evolution, because they play a crucial role in petal development. There are two lineages of B-class genes, which arose from a duplication event after the divergence of extant gymnosperms and angiosperms, but before the extensive diversification of the angiosperms (Stellari et al. 2004). *Arabidopsis* and *Antirrhinum* both contain one representative of each lineage. The *DEF*/*AP3* lineage is named after *APETALA3* from *Arabidopsis* and *DEF* from *Antirrhinum*, whereas the *GLO*/*PI* lineage contains, among others, the genes *PISTILLATA (Arabidopsis)* and *GLOBOSA (Antirrhinum)*.

In *Arabidopsis*, the B-class proteins AP3 and PI form a heterodimer that subsequently interacts with E-class proteins and A- or C-class proteins to promote the formation of the petals or stamens respectively (see Fig. 9.3). Loss-of-function mutants of either *AP3* or *PI* fail to establish petal and stamen identity, and specify sepals and carpels instead (Jack et al. 1992; Goto and Meyerowitz 1994). Similarly, the *Antirrhinum* B-class proteins DEF and GLO act together as heterodimers to determine petal and stamen identity (Tröbner et al. 1992). While *Antirrhinum* and *Arabidopsis* both contain only one representative from each lineage, other species

often contain more paralogous genes that belong to the same lineage, and may function in a redundant manner (Kramer and Jaramillo 2005; Rijpkema et al. 2006). An interesting duplication in the AP3/DEF lineage has occurred at the base of the core eudicots and gave rise to the *euAP3* and *TM6 (TOMATO MADS box gene 6)* lineages (Kramer et al. 1998). Proteins from both clades evolved completely different C-terminal motifs as a result of a frameshift mutation in the ancestor of the *euAP3* genes (Vandenbussche et al. 2003). *TM6*-like genes contain the original C-terminal motif and are present in basal eudicots, magnoliids monocots and basal angiosperms. *euAP3* genes contain the derived motif and are present exclusively in the core eudicots. While most core eudicots lost the ancestral TM6-like gene, species of the Solanaceae maintained both AP3/DEF paralogs. In *Petunia*, the *euAP3*-like gene *PhDEF* and the *TM6*-like gene *PhTM6* have functionally diverged and only partly execute the B function in a redundant manner (Rijpkema et al. 2006). *Petunia* also contains two representatives of the *GLO/PI* lineage, *PhGLO1* and *PhGLO2*. However, in contrast to the *AP3/DEF* genes, *PhGLO1* and *PhGLO2* have similar expression profiles and redundant activities (Rijpkema et al. 2006).

B-class genes have also been studied in non-eudicot species and revealed to be important for perianth and stamen formation in all investigated species (Kramer and Jaramillo 2005; Erbar 2007; Soltis et al. 2007a). In several lineages, duplications occurred in either the DEF/AP3 or the GLO/PI lineage, and paralogous genes function redundantly or partly redundantly. Grass flowers do not contain sepals or petals, but the lodicules, which facilitate the opening of the flower, are considered as a reduced perianth (Fornara et al. 2003). In rice, the *GLO/PI* paralogs *OsMADS2* and *OsMADS4* have separated functions in the second and third whorl. *OsMADS2* is exclusively involved in lodicule differentiation and growth, whereas *OsMADS4* is sufficient for stamen specification (Yadav et al. 2007). Although the B function is highly conserved among all angiosperms, changes in B-class gene expression revealed to be very important for the evolution of different flower morphologies, as discussed below in this chapter.

9.4.3 C Function

C-function genes are required for the specification of the reproductive organs in both angiosperms and gymnosperms and belong to the oldest clades of plant MADS box genes. In addition to the floral organ identity function, C-class genes are also essential for the maintenance of meristem determinacy. In *Arabidopsis*, the C function is fulfilled by a single gene, *AG*. Loss of *AG* function results in conversion of the third-whorled stamens into petals, and formation of indeterminate perianth whorls instead of carpels (Yanofsky et al. 1990). Most eudicotyledons contain two or more C-class genes as a result of a duplication event early in the evolution of the core eudicots. The duplicated genes were subjected to subfunctionalization and neofunctionalization and often diverged. As a result of this, the other *Arabidopsis* C-class genes, *SHATTERPROOF1 (SHP1)* and *SHP2*, do not fulfil a function in

stamen and carpel identities, but are active only in ovule formation (Favaro et al. 2003). In addition, these genes are involved in the formation of the dehiscence zone in *Arabidopsis* siliques. *Antirrhinum* contains two C-class genes, *PLENA (PLE)* and *FARINELLI (FAR)*, of which only *PLE* executes the full C function. *FAR* was subjected to subfunctionalization and is involved only in male fertility (Davies et al. 1999). Also petunia and cucumber each contain two genes that are highly homologous to *AG* (Tsuchimoto et al. 1993; Angenent et al. 1994; Kater et al. 1998). The petunia homologs *FLORAL-BINDING PROTEIN 6 (FBP6)* and *pMADS3* are both expressed in the stamen and carpel primordia. However, overexpression of *FBP6* does not result in homeotic conversion of sepals into carpels and petals into stamens, in contrast to overexpression of *pMADS3* (Kater et al. 1998), suggesting that *pMADS3* is the C-function gene in *Petunia*. The same study showed that overexpression of the *CUCUMBER MADS BOX GENE 1 (CUM1)* in petunia resulted in a full conversion of the sepals and petals, whereas overexpression of the highly homologous *CUM10* gene affected only the petals, which partially transformed into antheroid structures.

Studies in monocotyledons (Fornara et al. 2003), basal angiosperms (Soltis et al. 2007a) and gymnosperms (Zhang et al. 2004) revealed that C-class genes control reproductive organ identity in all seed plants, indicating that the mechanism of C-class function arose before the divergence of angiosperms and gymnosperms, and has been conserved during 300 million years of evolution (Zhang et al. 2004).

9.4.4 D Function

The initiation and specification of the ovule primordia on the placental wall inside the ovary is regulated by the D-class genes, in combination with the C- and E-class genes. D-class MADS box genes originated from a duplication event in the C-class lineage close to the base of the angiosperms, which gave rise to two lineages: genes that remained expressed in both male and female reproductive organs to fulfil the C function, and ovule-specific genes that specialized to execute the D function (Becker and Theissen 2003).

The D function was first described in *Petunia*, where loss of function of the paralogous genes *FBP7* and *FBP11* resulted in the conversion of ovules into spaghetti-like carpelloid structures (Angenent et al. 1995b). Ectopic expression of *FBP11* gave rise to ovule-like structures on the sepals and the petals (Colombo et al. 1995), indicating that *FBP11* is sufficient to promote ovule identity in the presence of the E-class proteins. The *Arabidopsis* gene *SEEDSTICK (STK)* is most homologous to the *Petunia* D-function genes, and is expressed exclusively in ovules. Ectopic expression of *STK* resulted in ovule development on carpelloid sepals (Favaro et al. 2003), indicating that *STK* fulfils the D function in *Arabidopsis*. However, the *stk* single mutant exhibits only minor defects in ovule development, as a result of redundancy with the C-class genes. Only in the *stk shp1 shp2* triple mutant is ovule formation impaired, and leaf-like or carpel-like structures are

formed instead of the ovules (Favaro et al. 2003; Pinyopich et al. 2003). This phenotype is enhanced if *AG* function is also lost, indicating that the C- and D-class genes act redundantly in specifying ovule identity in *Arabidopsis* (Pinyopich et al. 2003). Although the D-class genes of *Petunia* do not function redundantly with the C-class genes in the initiation of the ovules, it is likely that they act in combination with class C genes, because *FBP6* and *pMADS3* are also expressed in ovules, and D- and C-class proteins probably function in multimeric protein complexes (Ferrario et al. 2006).

Monocotyledons appear to have a homologous mechanism for ovule differentiation, because ovules are converted into carpelloid structures in the rice D-class mutant *osmads13*, and the D-class gene *ZAG2* from maize is expressed in ovule primordia (Dreni et al. 2007). However, although phylogenetic analysis revealed distinct C- and D-class genes in basal angiosperms, monocotyledons and eudicotyledons, gene expression in both lineages is variable and C-class genes are often also expressed in ovules, indicating that redundancy among C- and D-class genes may be common in ovule development (Zahn et al. 2006).

9.4.5 E Function

E-function genes encode MADS box transcription factors that are important for the formation of all floral organs. The E-class MADS box genes probably originated early in angiosperm evolution and have been isolated from basal angiosperms, monocotyledons and eudicotyledons (Becker and Theissen 2003; Soltis et al. 2007b). *Arabidopsis* contains four genes that belong to the E class, namely *SEPALLATA1 (SEP1), SEP2, SEP3* and *SEP4*. Single mutants of either of these genes exhibit only subtle phenotypes, but in *sep1/sep2/sep3* triple mutants, all floral organs are converted into sepals, similar to the phenotype of class B/C double mutants (Pelaz et al. 2000). If also *SEP4* is eliminated, all floral organs resemble leaf-like tissue (Ditta et al. 2004). The similarity between the *sep1/sep2/sep3* mutant and a B/C double mutant suggests that the *SEP* genes may function in regulating the expression of the B- and C-class genes. However, B and C homeotic genes are induced normally in the *sep* triple mutant and, reversibly, the *SEPs* are still expressed in B- or C-class mutants (Pelaz et al. 2000; Becker and Theissen 2003), demonstrating that these genes are not transcriptionally interlinked. Instead of regulating other floral organ genes, the E-function proteins play a critical role in the formation of higher-order complexes with the ABCD-function proteins. Without the E-function proteins, the B-function proteins AP3 and PI form a heterodimer that binds target DNA sequences, but is not able to activate transcription (Krizek and Fletcher 2005). In accordance with these data, ectopic expression of class B, E and *AP1* is sufficient to transform leaves into petals (Honma and Goto 2000).

The subfamily of *SEP*-like MADS box genes has undergone multiple duplication events throughout the evolution of flowering plants, resulting in paralogous genes with overlapping function in the majority of angiosperm species (Becker and

Theissen 2003). Because of the recent discovery of the E function and the high redundancy found among E-class genes, the E function has not yet been characterized in many species. In *Petunia*, six *SEP*-like genes were identified that probably all contribute to the E function to a certain extent (Rijpkema et al. 2006). Of these six genes, *FLORAL BINDING PROTEIN 2 (FBP2)* and *FBP5* have been demonstrated to promote higher-order complex formation with the *Petunia* B- and C-class proteins (Ferrario et al. 2003). The rice genes *OsMADS1, OsMADS5, OsMADS24, OsMADS45* and *OsMADS34* group together with the SEP genes in a phylogenetic tree, with *OsMADS24* and *OsMADS45* being the closest relatives to the *SEPs*. Expression analysis of both genes revealed expression in the lodicules, developing stamens and carpel primordia, suggesting that they fulfil the E function in rice (Fornara et al. 2003). The importance of the E-function genes in promoting all floral organ identities by enabling the formation of ternary complexes, and the apparent absence of *SEP* homologs in extant gymnosperms suggest that the E-function genes may have played a key role in the origin of the flower (Soltis et al. 2007b).

9.4.6 Variations on the Typical (A)BCDE Model

Arabidopsis, Antirrhinum and *Petunia* have a common flower body plan, consisting of four whorls that are distinctly separated. However, although the molecular mechanisms governing flower development are highly conserved, many species show variations on the typical flower plan, for example, by forming two whorls of petals or by the development of mono-sexual flowers. This variation can often be explained by a different expression of one or more of the homeotic genes involved in the ABCDE model. Especially shifts in the expression of B- or C-function genes have been shown to underlie flower diversity (Kramer and Jaramillo 2005). This phenomenon has been described in the ‘sliding boundary’ hypothesis (Kramer et al. 2003). This hypothesis explains, for example, the formation of an extra whorl of petals instead of sepals by the expansion of B-function expression to the first whorl, or the development of a mono-sexual male flower by a shift of B function to the inner whorl (Fig. 9.4a).

Basal angiosperms exhibit even a higher diversity in floral types, which cannot be explained by the ‘sliding boundary’ hypothesis alone. They often have many floral parts that are arranged in spirals, rather than in whorls, and the transition from one floral organ to another may occur gradually (Soltis et al. 2007a, b). Expression analysis of the B- and C-function genes in basal angiosperms revealed a broader pattern, with often a gradual reduction towards the borders of the expression domain. Rooted in these observations, the ‘fading borders’ model was developed based on gradients in the expression level of the homeotic genes (Fig. 9.4b; Soltis et al. 2007b). Since the ABCDE model with fixed borders is applicable only to the core eudicotyledons, the broad and sometimes gradual expression of the floral organ identity genes in the basal angiosperms is thought to be the ancestral state (Erbar 2007).

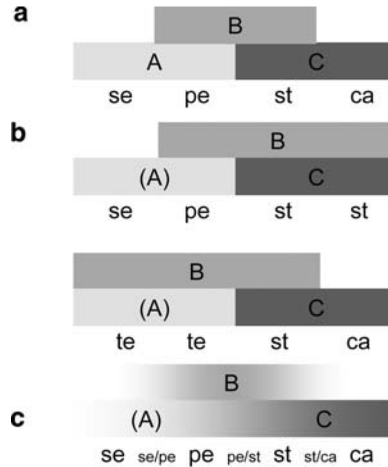

Fig. 9.4 Variations on the (A) BC model. **a** Classical ABC model as described for *Arabidopsis* (Coen and Meyerowitz 1991); **b** sliding boundary model (Kramer et al. 2003); **c** fading borders model (Soltis et al. 2007b). In the alternative models (**b** and **c**), the ambiguous status of the A function is illustrated by placing the letter A within brackets. Abbreviations: se, sepals; pe, petals; st, stamens; ca, carpels; te, tepals

9.5 Autoregulatory Mechanisms

MADS box transcription factors regulate transcription of target genes by binding a consensus sequence known as the CArG box, which represents the consensus sequence CC(A/T)6GG. Besides the control of downstream target genes, MADS box factors often regulate their own expression or the expression of family members by autoregulatory mechanisms. Although the FM identity genes *LFY, AP1, CAL* and *FUL* are initially responsible for the activation of the floral organ identity genes, the floral organ identity genes themselves subsequently influence and refine their expression patterns (Krizek and Fletcher 2005). Studies in *Arabidopsis*, *Antirrhinum* and *Petunia* revealed that the expression of the B-class genes is maintained by complexes composed of B proteins (Schwarz-Sommer et al. 1992; Angenent et al. 1995a; Samach et al. 1997). Positive autoregulatory loops have also been reported for AG and the homeotic proteins with which it interacts (SEP3, AP3 and PI; de Folter et al. 2005; Gomez-Mena et al. 2005), and for the *Petunia* proteins FBP2 and FBP11, which induce the expression of *FBP7, FBP11* and *FBP6* when overexpressed together (Ferrario et al. 2006). Regulation via positive and negative (auto) regulatory loops provides a robust mechanism by which the expression of genes can be maintained until the transition to another phase requires a rapid decline.

9.6 Other Genes Involved in Floral Organogenesis

The identity of the floral organs is determined by the homeotic ABCDE genes, but other genes have also been found to play important roles in floral organ formation. Except for the target genes of the ABCDE genes, a number of cadastral genes,

involved in defining the boundaries of the floral whorls, are essential for normal flower development. The *Arabidopsis* proteins LEUNIG (LUG) and SEUSS (SEU) repress the expression of AG in the outer two whorls, possibly in a complex with AP2. A similar function has been reported for *STYLOSA (STY)*, the *Antirrhinum LUG* ortholog (Motte et al. 1998; Sridhar et al. 2004; Krizek and Fletcher 2005). The inner boundary of the B function in *Arabidopsis* flowers is regulated by *SUPERMAN (SUP)*, a C2H2 zinc-finger protein. *SUP* determines the border between the third and the fourth whorl by regulating the balance of cellular proliferation in the inner two whorls and repressing B gene expression in the fourth whorl (Yun et al. 2002; Krizek and Fletcher 2005). An *Antirrhinum* ortholog of *SUP* has not been identified, but the *Petunia* homolog of *SUP*, *PhSUP1*, is similarly involved in the specification of the boundary between whorl 3 and 4, indicating that *SUP* function is conserved in angiosperms (Nakagawa et al. 2004).

The F-box protein *UFO* has been identified as an FM identity gene in *Arabidopsis*, and plays a role in floral organ formation by activating the B-function genes *AP3* and *PI*. In addition, *UFO* has an early cadastral function in flower development by regulating the position of the primordia of whorls 2, 3 and 4 (Laufs et al. 2003). The *Antirrhinum* ortholog *FIMBRIATA (FIM)* plays a similar role in mediating between meristem and organ identity genes, although some differences in the functions and genetic interactions of *UFO* and *FIM* demonstrate that divergence has occurred during evolution (Ingram et al. 1995).

9.7 Targets of the Floral Organ Identity Genes

The floral organ identity genes induce the formation of the floral organs by activating a set of downstream genes involved in the specification of tissue and cell identities. Some downstream targets of the ABCDE genes have been identified in *Arabidopsis* by genetic studies, micro-array assays or chromatin immunoprecipitation (ChIP). This includes *NAC-LIKE, ACTIVATED BY AP3/PI (NAP)*, which functions in the transition from cell division to cell expansion in stamens and petals (Sablowski and Meyerowitz 1998), and *SPOROCYTLESS (SPL)*, which is activated by *AG* and regulates ovule patterning and early microsporogenesis (Ito et al. 2004). Other genes that are known to be activated by *AG* are *SHP1* and *SHP2*, which play a role in the formation of the dehiscence zone in *Arabidopsis* siliques (Liljegren et al. 2000). Although to date not many target genes of the floral organ identity genes have been characterized, the current available techniques allow a high-throughput analysis for species of which the genome has been sequenced, and many new data can be expected soon. Target gene identification is the next step in unravelling the regulatory pathway that begins with the homeotic genes and ends with the formation of the flower.

9.8 Summary

The analysis of floral homeotic mutants from *Arabidopsis, Antirrhinum* and *Petunia* resulted in the formulation of the ABCDE model for floral organ development, which contains almost exclusively MADS box genes. The model describes the combinatorial action of the functional classes A, B, C, D and E in the establishment of the floral organ identities. The ABCDE genes are highly conserved in angiosperms, and have been found in all angiosperm species studied so far. However, not all orthologs appear to have similar functions in floral organ initiation, and especially the status of the A function is still ambiguous. In contrast, the B- and C-function genes have been functionally conserved in a broad range of angiosperm species, including monocots and basal angiosperms. Shifts in the expression of B- or C-function genes have been shown to underlie flower diversity and have led to the development of alternative models, like the ‘fading borders’ model.

The key genes that regulate floral organ initiation and development have been identified in *Arabidopsis* and several other model species in the past decades. The next step in unravelling the regulatory pathways controlling the formation of the flower is the identification of the target genes that are regulated by the MADS box protein complexes. More diversity in the underlying pathways are expected, which should reflect the diversity in floral shapes and sizes among angiosperm flowers.

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Chapter 10
Control of Flower Development

H. Yamashita and Y. Komeda

10.1 Introduction

The transition from vegetative growth to flowering occurs at the shoot apical meristem (Simpson et al. 1999; Parcy 2005). Floral induction causes an apical meristem to produce flowers, which consist of a complex array of specialized structures (Zeevaart 1976; Bernier 1998). Flowering is regulated by signals from endogenous and external sources. Endogenous signals include circadian rhythms, developmental stage and hormones, while external signals comprise day length and temperature.

It has been well documented that floral stimuli are translocated from the leaves to the shoot apical meristem (Garner and Allard 1920; Evans 1971; Yanovsky and Kay 2002; Searle and Coupland 2004). The interaction of these endogenous and external signals enables the plant to synchronize its reproductive development with the environment (Fig. 10.1). Morphological changes first occur during the transition from the vegetative to reproductive stage (Lang 1952; Weigel 1995). When reproductive development is initiated, the vegetative meristem is transformed into the primary inflorescence meristem that, in turn, produces an elongated inflorescence axis bearing cauline leaves and flowers. The axillary buds of cauline leaves develop into secondary inflorescence meristems. The inflorescence meristem grows indefinitely and exhibits indeterminate growth. Floral meristems give rise to flowers, which are formed by determinate growth.

H. Yamashita
Kyoto Prefectural University Faculty of Life and Environmental Sciences, Laboratory of Plant Molecular Biology, Shimogamo-nakaragi-cho, Sakyo-ku, Kyoto 606-8522, Japan

Y. Komeda
The University of Tokyo, Graduate School of Science, Department of Biological Sciences, Laboratory of Plant Science, Hongo, Bunkyo-ku, Tokyo113-0033, Japan
e-mail: komeda-y@biol.s.u-tokyo.ac.jp

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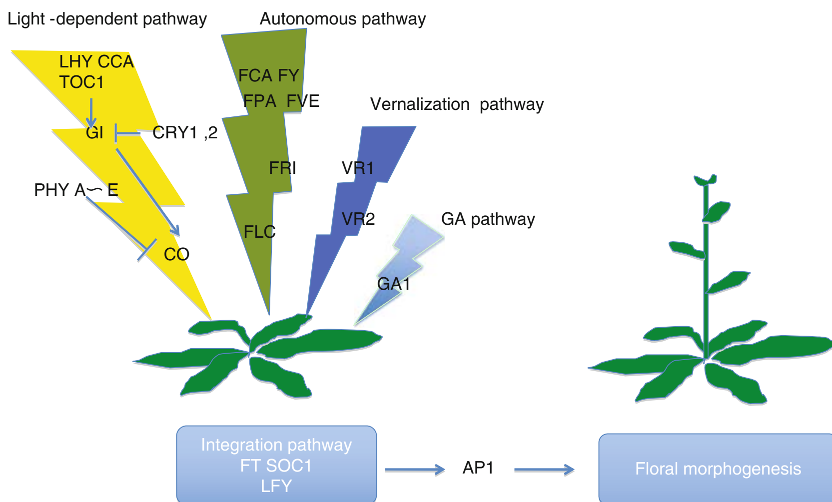

Fig. 10.1 The genetic pathways of flowering in *Arabidopsis thaliana*. *Arrows* indicate positive interaction, and *T-lines* negative interaction

10.2 Regulation of Floral Organ Development

Floral meristems give rise to four different types of floral organs, namely sepals, petals, stamens and carpels. In the wild-type *Arabidopsis* flower, these organs are positioned in four successive whorls, with four sepals in the first whorl, four petals in the second whorl, six stamens in the third whorl and two carpels in the fourth whorl.

10.2.1 Genes Associated with Floral Development

Three types of genes regulating floral development have been identified. These include meristem identity genes, floral organ identity genes and cadastral genes (McSteen et al. 1998). Meristem identity and floral organ identity genes encode transcription factors (Busch et al. 1999). These proteins control the formation of floral organs. Cadastral genes are regulators of the floral organ identity genes. Meristem identity genes act on immature primordia to become floral meristems (Simon et al. 1996). In snapdragon, mutation of the meristem identity gene *FLORICAULA* (*FLO*) resulted in the formation of an inflorescence that did not produce flowers, indicating that the *FLO* gene controls the determination of floral meristem identity. Several floral meristem identity genes have been identified in *Arabidopsis*; these genes are *SUPPRESOR OF CONSTANS 1* (*SOC1*), *APETALA1* (*AP1*) and *LEAFY* (*LFY*). *SOC1* acts as a master switch, initiating floral development and triggering *LFY* expression. In turn, *LFY* activates *AP1*, the expression of which

stimulates that of *LFY* (Wagner et al. 1999). The genes that determine floral organ identity were discovered as floral homeotic mutants. The homeotic genes encode transcription factors binding to DNA. Most of these genes possess sequences known as MADS box, which encodes proteins with the MADS domain. Many of the genes that determine floral organ identity are MADS box genes, for example, snapdragon *DEFICIENS*, *Arabidopsis AGAMOUS* (*AG*), *PISTILLATA* (*PI*) and *APETALA3* (*AP3*).

In dicotyledonous plants, the *apetala2* (*ap2*) mutant produces flowers comprising only carpels and stamens, without sepals and petals. This is because sepals are converted into carpels, and petals into stamens. In *ap3* or *pi* mutants, the flowers contain sepals instead of petals in the second whorl, and carpels instead of stamens in the third whorl. These homeotic genes have been classified into class A, B and C. Class A genes, including *AP1* and *AP2*, are known to regulate organ identity in the first and second whorls. Class B genes, such as *AP3* and *PI*, control organ determination in the second and third whorls. As for the class C genes, such as *AG*, they regulate organ determination in the third and fourth whorls. With respect to function, class A specifies sepals, while both class A and B regulate the formation of petals; class B and C are involved in the regulation of stamen formation. The function of class C alone has been shown to regulate carpel formation and determinate developmental.

10.2.2 Photoperiodism

Photoperiodism makes it possible for an event to occur at a particular time (Garner and Allard 1920, 1923; Mockler et al. 2003). One of the important plant responses controlled by day length is the initiation of flowering. Plant photoperiodic responses use the same photoreceptors. There are two main photoperiodic responses, namely short-day (SD) and long-day (LD). SD and LD plants flower only under SD and LD conditions respectively. Flowering of an LD plant is promoted when the day length exceeds a given duration, which is referred to as critical day length. In contrast, promotion of flowering in SD plants requires a day length that is less than the critical day length. LD plants delay flowering until the critical day length is reached, whereas SD plants flower when the days are shorter than the critical day length. Day-neutral plants have no photoperiodic preference for flowering, the latter being controlled by autonomous regulation.

The photoperiodic signal is perceived by the leaves. Treatment of a single leaf of a SD plant with short photoperiods is sufficient to cause the formation of flowers, even when the rest of the plant is exposed to LD. In response to photoperiod, the leaf transmits a signal that regulates the transition to flowering at the shoot apex. Photoperiodic induction is the photoperiod-regulated process that occurs in leaves and results in the transmission of a floral stimulus to the shoot apex. Photoperiodic induction can take place in a separated leaf, and the induced leaf can cause flowering in non-induced plants. The leaf floral signal is translocated via the phloem to the

shoot apical meristem and promotes floral induction. When the phloem translocation is blocked, it inhibits flowering by preventing the movement of the floral stimulus from the leaf. Furthermore, a night break treatment, in which the dark period is interrupted by a short exposure to light, is effective in preventing flowering, as in *Pharbitis*.

An *Arabidopsis*, the *CONSTANS* (*CO*) gene, which encodes a zinc finger transcription factor regulating the transcription of other genes, has been shown to promote flowering (Robson et al. 2001; Valverde et al. 2004; Wenkel et al. 2006). The *co* mutant is not responsive to photoperiodic-induced flowering, and *CO* expression is controlled by the circadian clock. In a long day, flowering can be induced by the accumulation of CO protein, which is regulated at the post-transcriptional level. In darkness, CO is tagged with ubiquitin and rapidly degraded by 26S proteasome. However, light appears to enhance the stability of the CO protein, which is accumulated during the day. Flowering is also associated with phytochrome (e.g. *PHYA*, *PHYB*) and cryptochrome (e.g. *CRY2*) genes. Mutations in the *PHYA* gene have been shown to delay flowering in *Arabidopsis*. Blue light promotes flowering. It has been observed that the *cry2* mutation caused a delay in flowering and inability to perceive an inductive photoperiod. Moreover, gain of function of *CRY2* has resulted in early flowering, indicating that cryptochromes regulate flowering directly by stabilizing the CO protein. Results from several lines of study showed that *PHYB* signalling enhances CO degradation in the morning, whereas in the evening cryptochromes and *PHYA* antagonize this degradation process, thereby facilitating the synthesis of CO protein. CO has been shown to promote flowering by stimulating the expression of downstream genes such as *FT* and *SOC1* (An et al. 2004; Yoo et al. 2005). Overexpression of *FT* resulted in rapid flowering, regardless of the photoperiod. Furthermore, *FT* expression can be elevated during the inductive photoperiod, showing that *FT* is a strong promoter of flowering. Like FT, *CO* also exhibits a similar pattern of circadian mRNA accumulation.

10.2.3 Vernalization

Vernalization is the process whereby flowering is promoted by a cold treatment. Without the cold treatment, plants that require vernalization show delayed flowering. The vernalization process regulates specific target genes. For example, the *FLOWERING LOCUS C* (*FLC*) gene, which acts as a repressor of flowering, is highly expressed in non-vernalized shoot apical meristems (Searle et al. 2006).

10.2.4 Florigen

It has been known for some time that photoperiodically induced leaves could produce a biochemical signalling compound that is transported to a distant target

region to promote flowering. This signalling compound is called flowering hormone or florigen. The existence of florigen has been demonstrated by grafting a plant with a leaf or shoot originated from a photoperiodically induced donor plant. Results from several lines of study showed that *FT* mRNA could be translated into protein in the apex, where the FT protein formed a complex with the transcriptional factor, FD (Kardailsky et al. 1999; Abe et al. 2005; Jaeger et al. 2006; Corbesier et al. 2007). The FD and FT complex activated downstream target genes such as the *SOC1*, *AP1* and *LFY* floral homeotic genes (Wigge et al. 2005).

10.3 Genetic Network of Flowering Control

There are four pathways corresponding to flowering control in *Arabidopsis* and other model plants (Koornneef et al. 1991, 1998).

10.3.1 Light-Dependent Pathway

Phytochrome is a plant photoreceptor with two interconvertible forms, Pr and Pfr (Chen et al. 2004). Pr molecules are synthesized to absorb maximally in red light (R) and can be photo-transformed to Pfr with maximum absorbance in far-red light (FR). Higher plants possess several phytochromes encoded by divergent genes (*PHYA* through *PHYE* in *Arabidopsis thaliana*; Quail et al. 1995). Phytochrome A (coded by *PHYA*) mediates two photobiologically distinct types of response, namely very-low-fluence response (VLFR) and high-irradiance response (HIR; Casal et al. 2003).

In *Arabidopsis*, a blue/UV-A photoreceptor, CRYPTOCHROME 2 (by the *CRY2* gene), and a red/far-red photoreceptor, PHYTOCHROME B (by the *PHYB* gene), are the two major photoreceptors that control flowering (Guo et al. 1999). The light stimuli for flowering regulation are perceived by the leaves (Roden et al. 2002). *PHYB* expression in mesophyll, but not in vascular bundles, suppressed *FT* expression in vascular bundles. CRY2 in leaves perceives light stimuli to regulate flowering. Transgenic *Arabidopsis* lines expressing a *CRY2-green fluorescent protein* (*GFP*) fusion under the control of organ/tissue-specific promoters in a *cry2*-deficient mutant background have been analyzed. The results showed that *CRY2-GFP* was expressed in vascular bundles, but not in the epidermis or mesophyll, and rescued the late-flowering phenotype. Expression of *CRY2-GFP* in vascular bundles promoted *FT* expression only in vascular bundles. These results are contrary to that of PHYB and CRY2, indicating that *FT* expression may be regulated in a cell-autonomous manner.

Of the five phytochromes identified in *Arabidopsis*, phytochrome B (encoded by the *PHYB* gene) plays the most important role in shade-avoidance responses, but the mechanism whereby PHYB regulates flowering in response to altered ratios of

red to far-red light is not clear. *PFT1* (*PHYTOCHROME AND FLOWERING TIME 1*), encoding a nuclear protein, acts in a PHYB pathway and induces flowering in response to suboptimal light conditions. PFT1 functions downstream of the PHYB pathway to regulate *FT* expression. This provides evidence for the existence of a light-quality pathway that regulates flowering time.

A photomorphogenic mutant, *hypersensitive to red and blue 1* (*hrb1*), showed late flowering and attenuated expression of *FT* in both long days and short days. Transgenic plants that overexpressed the full-length *HRB1* or its C-terminal half flowered early and accumulated more *FT* transcripts under short-day conditions. The transgenic plants also displayed hyposensitive de-etiolation phenotypes, and the transgene expression in these phenotypes required the action of PIF4. The double mutant of *hrb1*/*cry2* exhibited a flowering phenotype and an *FT* expression pattern similar to those of *hrb1* under long-day conditions, suggesting that HRB1 may function downstream of CRY2 under these conditions. In contrast, *hrb1*/*phyB-9* had a flowering phenotype and an *FT* expression pattern similar to those of *phyB-9* over both long days and short days, indicating a modulatory role of HRB1 in the flowering pathway mediated by PHYB. Overexpression of *HRB1* did not affect the expression of the central clock oscillators *TOC1* and *CCA1*. It is therefore speculated that HRB1 may serve as a signalling protein regulating *FT* expression downstream of red and blue light perception.

In plants, the shift from vegetative growth to floral development is regulated by red/far-red light receptors (phytochromes) and blue/ultraviolet A light receptors (cryptochromes). While a mutation in the *CRY2* gene is allelic to the late-flowering mutant *fha*, flowering in the *cry2*/*fha* mutant is partially responsive to photoperiod. Cryptochrome 2 is a positive regulator of the flowering-time gene *CO*, and its expression has been shown to be regulated by photoperiod. The function of CO can be repressed by PHYB. The implication of PHYB in flowering has been shown in the *PHYB* loss-of-function mutants, which displayed early flowering, whereas early flowering was not observed in *co*/*phyB* double mutants. Another cryptochrome gene, *CRY1*, functions cooperatively with the *CRY2* gene in flowering. Analysis of flowering in *cry2* and *phyB* mutants in response to different wavelengths of light indicated that flowering is regulated by the antagonistic actions of PHYB and CRY2.

In *Arabidopsis*, the flowering time is regulated through the circadian clock, which measures day length and modulates the photoperiodic CO-FT output pathway in accordance with the external coincidence model (Reeves and Coupland 2001; Suárez-López et al. 2001; Ni 2005). A set of mutants, including the early-flowering *toc1*/*cca1*/*lhy* triple mutant, showed that CCA1 and LHY acted redundantly as negative regulators of the photoperiodic flowering pathway. The partially redundant CCA1/LHY functions are dependent largely on the upstream *TOC1* gene that serves as an activator. For this linkage, the phase control of certain flowering-associated genes, such as *GI*, *CDF1* and *FKF1*, appears to be crucial. Furthermore, the genetic linkage between *TOC1* and *CCA1*/*LHY* is compatible with the negative and positive feedback loop, which may serve as core of the circadian clock. The circadian clock may open an exit for a photoperiodic output pathway during the daytime.

The processed signal is transmitted to the *GI* gene, thereby activating *CO* expression. The "light" signal from the environment sets the circadian clock to regulate multiple physiological processes for optimal rhythmic growth and development. One such process is the control of flowering time by photoperiod perception in plants. In *Arabidopsis*, the flowering time is determined by the correct interconnection of light input and signal output by the circadian clock. The identification of additional clock proteins will help to dissect the complex nature of the circadian clock in *Arabidopsis*. LWD1/LWD2 is the new clock protein involved in photoperiod control (Wu et al. 2008a). The *lwd1lwd2* double mutant displayed an early-flowering phenotype, which is attributed to the significant phase shift of *CO* and an increased expression of *FT* before dusk. Under entrainment conditions, the expression phase of oscillator (*CCA1*, *LHY*, *TOC1* and *ELF4*) and output (*GI*, *FKF1*, *CDF1*, *CO* and *FT*) genes in the photoperiod pathway shifts approximately 3 h forwards in the *lwd1lwd2* double mutant. Both the oscillator and output (*CCR2* and *CAB2*) genes have a short period length in the *lwd1lwd2* double mutant. LWD1/LWD2 proteins function in close proximity to or within the circadian clock for photoperiodic flowering control. CO has the function of integrating the light pathway (Samach et al. 2000). The *co* mutants have been shown to display late flowering under LD conditions. The *CO* gene shows homology to the Zn-finger domain proteins of a transcriptional factor.

10.3.2 Gibberellin Pathway

Gibberellic acid (GA) plays a crucial regulatory role in plant growth and development. It also acts as florigen in LD plants (Wilson et al. 1992). Late-flowering *gi* mutants are defective for a membrane protein with a membrane-spanning region. These mutants are also defective for the expression of *CCAI* and *LHY* genes. The GI protein has been shown to express with a circadian rhythm. *A. thaliana ga1-3* plants do not flower and LFY activity is lost under SD conditions. However, the mutant overexpressing *LFY* promoted flowering even under SD conditions.

10.3.3 Autonomous Pathway

Both environmental and developmental (internal) factors are involved in promoting flowering (Ruiz-García et al. 1997; Liu et al. 2007). The early-flowering ecotypes (e.g. Columbia, Landsberg *erecta* and WS) have been shown to possess mutations in the *FRIGIDA* (*FRI*) gene, indicating that *FRI* is associated with late flowering. *FRI* encodes a coiled-coil domain protein with 619 amino acid residues. It has been suggested that coiled-coil domains may have a regulatory function of *FLC*, which encodes a MADS box protein family and plays a key role in vernalization. FRI is a positive regulator of the FLC repressor for flowering.

The mutants with loss of function of *LD* or *FCA* genes were late flowering. *LD* encodes a protein with the nuclear-localizing signal and shows homology to plant DNA-binding protein and mammalian transcription domain, while *FCA* encodes an RNA-binding protein with the domain of RRM-protein interaction (Page et al. 1999; Bäurle et al. 2007). The domain of FCA has been characterized as a flowering-time regulator and is required for RNA-mediated chromatin silencing. Furthermore, FCA promotes DNA methylation and the gene has been shown to transcribe four types of the message, namely alpha, beta, gamma and delta. The gamma message is the only functional message and when expressed strongly in transgenic plants, the latter showed early flowering. In addition to FCA, other genes with similar functions include *FVE*, *FPA* and *FY*, which repress *FLC*. An insertional mutation of *FLC* did not cause early flowering, leading to speculation that *FLC* is not the only regulator to downstream genes.

AtGRP7 has been shown to be a flowering-time gene that encodes an RNA-binding protein. The latter is part of a circadian slave oscillator in *A. thaliana*. It negatively regulates its own mRNA, and affects the transcript levels of other genes. In a T-DNA insertional mutant, *atgrp7-1*, the plant flowered later than wild type under both LD and SD conditions. Independent RNA interference (RNAi) lines with reduced levels of AtGRP7 and AtGRP8 also displayed late flowering, particularly under short photoperiods. Consistent with the retention of a photoperiodic response, the transcript encoding the key photoperiodic regulator CO oscillates with the similar pattern between *atgrp7-1* mutant and wild-type plants. In both the RNAi lines and the *atgrp7-1* mutant, the transcript levels for the floral repressor *FLC* are elevated. Conversely, in transgenic plants ectopically overexpressing *AtGRP7*, the transition to flowering is accelerated mainly under SD, with a concomitant reduction in *FLC* abundance. However, the late-flowering phenotype of RNAi lines could be suppressed by introducing the *flc-3* loss-of-function mutation. These results suggest that *AtGRP7* promotes floral transition at least in part by down-regulating *FLC*. Furthermore, vernalization has been shown to override the late-flowering phenotype. Retention of both the photoperiodic and vernalization responses is the feature of autonomous pathway mutants, suggesting that *AtGRP7* is a novel member of the autonomous pathway.

10.3.4 Vernalization Pathway

Both *VRN1* and *VRN2* genes have been identified to be associated with vernalization. *VRN2* encodes a protein with homology to the PcG protein. It represses *FLC* expression and may play a role in keeping the FLC-chromatin state. FLC has been shown to participate in the autonomous and vernalization pathways. Other flowering mutants have been used for studying the time control of flowering, for example, embryonic flower (*emf*) mutants, which display early flowering, but also showed abnormal development, such as late germination and poor cotyledon expansion. The *EMF* genes function to maintain the repressible stage of flowering (Moon et al.

2003). While *EMF1* encodes a 121-kD transcriptional factor, *EMF2* encodes a 71-kD Polycomb group (PcG) protein containing a zinc finger motif and a cluster of tryptophan- and methionine-rich sequences. Both *EMF1* and *EMF2* play an important role in the flowering pathway.

In *Arabidopsis*, the WNK (with no lysine kinase) protein kinase-like genes have been identified. These genes consist of nine members, designated as *AtWNK1* to *AtWNK10* (Wang et al. 2008). T-DNA knockout mutations in *AtWNK2*, *AtWNK5* and *AtWNK8* genes caused early flowering. In contrast, a T-DNA knockout *wnk1* mutant resulted in much delayed flowering time. In addition, the transcript levels of several genes in the photoperiod pathway for flowering, such as *ELF4*, *TOC1*, *CO* and *FT*, were altered in *atwnk* mutants. These results show that the *Arabidopsis WNK* gene family regulates flowering time by modulating the photoperiod pathway. In petunia (*Petunia hybrida*), the ubiquitous expression of some homolog genes resulted in early flowering, and the inflorescence was transformed into a solitary flower and leaves into petals (Souer et al. 2008).

The expression of floral meristem identity (FMI) genes is regulated in petunia via transcription of a distinct gene, *DOUBLE TOP* (*DOT*), a homolog of *UNUSUAL FLORAL ORGANS* (*UFO*) from *Arabidopsis*. Mutation of *DOT* or its tomato (*Solanum lycopersicum*) homolog, *ANANTHA*, abolishes FMI. Ectopic expression of *DOT* or *UFO*, together with *LFY* or its homolog *ABERRANT LEAF AND FLOWER* (*ALF*), activates genes in petunia seedlings required for identity or outgrowth of organ primordia. *DOT* interacts physically with *ALF*, suggesting that it activates *ALF* by a post-translational mechanism. A wider role than previously thought for *DOT* and *UFO* in the patterning of flowers indicates that the different roles of *LFY* and *UFO* homologs in the spatiotemporal control of floral identity in distinct species result from their divergent expression patterns.

The petunia cytokinin-binding protein PETCBP exhibits high sequence similarity to *S*-adenosyl-L-homocysteine hydrolase (SAHH; Godge et al. 2008). Transgenic petunia plants expressing antisense SAHH displayed profuse branching, delayed flowering and shoot bud induction from leaf explants in vitro. Homologs have also been isolated from *A. thaliana* (*homology-dependent gene silencing 1*, *HOG1*) and *Orzya sativa* (*OsCBP*). *Arabidopsis HOG1* showed high-affinity cytokinin-binding activity and modified plant architecture, similar to *PETCBP*. Transgenic *Arabidopsis* plants overexpressing *HOG1* showed early flowering. In contrast, antisense plants were characterized by profuse branching, delayed flowering, increased leaf size and higher seed yield. These results suggest that genetic manipulation of this cytokinin-binding protein or its orthologs may be used for improvement of crop biomass and seed yield.

The rice E3 ligase gene, *SPOTTED LEAF11* (*SPL11*), which negatively regulates programmed cell death and disease resistance, has been shown to regulate flowering by interaction with *SPIN1* (*SPL11-interacting protein1*; Vega-Sánchez et al. 2008). SPIN1 is a signal transduction and activation of an RNA family member, which binds RNA and DNA in vitro and interacts with SPL11 in the nucleus. The *spl11* mutants displayed delayed flowering under LD conditions. *SPIN1* overexpression resulted in late flowering independently of day length.

Expression analyses of flowering marker genes in these lines suggested that SPIN1 repressed flowering by down-regulating the flowering promoter gene *Heading date3a* (*Hd3a*) via *Hd1*-dependent mechanisms in SD and by targeting *Hd1*-independent factors in LD. Both *SPIN1* and *SPL11* are regulated diurnally in opposing phases. While SPL11 negatively regulates *SPIN1* transcript levels, *SPL11* expression is affected by SPIN1. Furthermore, coincidence of high accumulation of *SPIN1* mRNA with the light in the morning and early evening is needed for flower repression. SPIN1 is mono-ubiquitinated by SPL11, suggesting that it may not be targeted for degradation. SPIN1 acts as a negative regulator of flowering that itself is negatively regulated by SPL11, possibly via ubiquitination.

The switch from vegetative to reproductive growth is marked by the termination of vegetative development and the adoption of floral identity by the shoot apical meristem (SAM). This process is called floral transition. The two maize MADS box (*ZMM*) genes *ZMM4* and *ZMM15*, associated with floral induction, have been mapped to duplicated regions of chromosomes 1 and 5, and are linked to the neighbouring MADS box genes *ZMM24* and *ZMM31* respectively (Danilevskaya et al. 2008). This gene order is syntenic with the vernalization1 locus responsible for floral induction in winter wheat (*Triticum monococcum*) and similar loci in other cereals. Analyses of temporal and spatial expression patterns indicated that the duplicated pairs *ZMM4-ZMM24* and *ZMM15-ZMM31* are coordinately activated after the floral transition in early-developing inflorescences. More detailed analyses revealed that *ZMM4* expressed initially in leaf primordia of vegetative shoot apices and that later expression increased within the elongating meristems acquiring inflorescence identity. Expression analysis in late-flowering mutants positioned all four genes downstream of the floral activators *indeterminate1* (*ID1*) and *delayed flowering1* (*dlf1*). Overexpression of *ZMM4* in transgenic maize resulted in early flowering and suppressed the late-flowering phenotype of both *id1* and *dlf1* mutants. It has been speculated that *ZMM4* may play a role in both floral induction and inflorescence development.

In *Arabidopsis*, the *SET2* homologs are involved in flowering time (Xu et al. 2008). Histone lysines can be mono-, di- or tri-methylated, providing an ample magnitude of epigenetic information for transcriptional regulation. The degree of H3K36 methylation is regulated by distinct methyltransferases. Each of the *SET2* homologs, *SDG8* and *SDG26*, is capable of methylating oligonucleosomes in vitro and both proteins are localized in the nucleus. It has been reported that loss-of-function *sdg8* mutants displayed an early-flowering phenotype, but the loss-of-function *sdg26* mutants showed a late-flowering phenotype. These findings are in line with the results showing that several MADS box flowering repressors are down-regulated by *sdg8* but up-regulated by *sdg26*. The *sdg8*, but not *sdg26*, mutant displayed a markedly reduced level of both di- and trimethyl-H3K36 and an increase in the level of monomethyl-H3K36, indicating that SDG8 is specifically required for di- and trimethylation of H3K36. H3K36 with di- and trimethylation, but not monomethylation, has been correlated with transcription activation. *SDG8* and *VIP4*, which encode a component of the *PAF1* complex, act independently and synergistically in transcription regulation. The deposition of H3K36 methylation is

finely regulated, possibly to cope with the complex regulation of growth and development in higher eukaryotes.

Another MADS box gene associated with flowering is *AGL19*, which is regulated by PcG in *Arabidopsis*. The PcG proteins form a cellular memory by maintaining the developmental regulators in a transcriptionally repressed state (Schönrock et al. 2006). *AGL19* is partly responsible for the early-flowering phenotype of *clf* mutants and its expression is maintained at low levels by the PcG proteins, such as MSI1, CLF and EMF2. The *AGL19* chromatin is strongly enriched in trimethylation of Lys 27 on histone H3 (H3K27me3), but not in H3K9me2. Repressive H3K27me3 marks were reduced by decreased levels of CLF or MSI1 and by a prolonged cold period, suggesting that MSI1 and CLF repress *AGL19* in the absence of coldness. Ectopic expression of *AGL19* strongly accelerates flowering, whereas *agl19* mutants showed a decreased response to vernalization and promotion of flowering by a prolonged cold period. Epistasis analyses revealed that *AGL19* worked in the poorly characterized *FLC*-independent

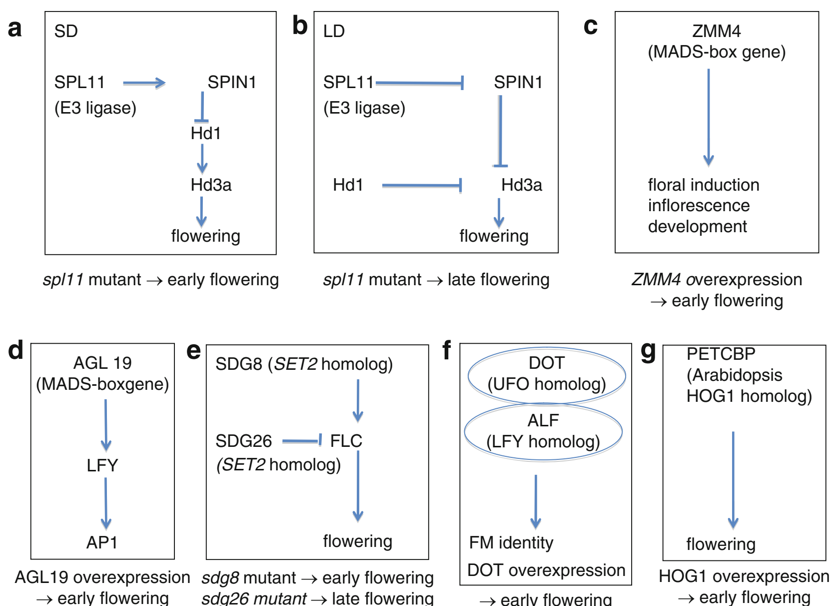

Fig. 10.2 Schematic of flowering-time mutant. *Arrows* indicate positive interaction, and *T-lines* negative interaction. **a** SPIN1 interacts with SPL11. SPIN1 represses Hd1 under short-day (SD) conditions. SPIN1 down-regulates the flowering promoter gene Hd3a. Spl11 mutants induce early flowering under SD conditions. **b** SPLL11 negatively regulates Spin1 transcript levels. SPIN1 represses Hd3a by targeting Hd1-independent factors. Spl11 mutants delay flowering under SD conditions. **c** Overexpression of ZMM4 promotes floral transition. **d** AGL19 activates LFY and AP1. Overexpression of AGL19 promotes flowering. **e** SDG8 activates transcription of FLC. SDG26 represses FLC expression. Loss of function of SDG8 exhibits early flowering. Loss of function of SDG26 causes late flowering. **f** DOT interacts with ALF. DOT activates ALF and controls FM identity. Overexpression of DOT causes early flowering. **g** Overexpression of HOG1 induces early flowering. PETCBP antisense lines delay flowering

vernalization pathway and did not require the function of *SOC*. In this pathway, prolonged coldness appears to relieve *AGL19* from PcG repression by a mechanism that requires VIN3 but not VRN2. Elevated *AGL19* levels activate *LFY* and *AP1*, leading to flowering. The genes characterized are summarized in Fig. 10.2.

10.4 Perspectives

Late- or early-flowering mutants have been used to study time control of flowering. A non-flowering mutant has been isolated recently. Although many flowering pathways have been integrated, the system has redundancy in *Arabidopsis*, because flowering can be delayed but not blocked completely in flowering mutants. Recently, a novel gene, *Rice Indeterminate 1* (*RID1*), which acts as the master switch for the transition from the vegetative to reproductive phase, has been identified in rice. *RID1* encodes a Cys-2/His-2-type zinc finger transcription factor. An *RID1* knock-out mutant shows a never-flowering phenotype (Wu et al. 2008b), indicating that *RID1* is involved in regulating flowering in the photoperiod pathway. *RID1* controls the phase transition and initiation of floral induction, but it seems to be independent of the circadian clock. The ortholog of *RID1* has not been identified in *Arabidopsis*. Thus, considering how *RID1* regulates flowering may lead to a better understanding of the regulatory mechanism of floral induction.

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Chapter 11
Development and Function of the Female Gametophyte

N. Sánchez-León and J.-P. Vielle-Calzada

11.1 Introduction

The life cycle of flowering plants is characterized by the alternation of two distinct generations. The sporophyte is the predominant generation and is represented by all the vegetative organs. The alternation from sporophyte to gametophyte involves the transition from a massive plant body to a structure composed of a small number of organized and highly specialized haploid cells. The anthers produce the male gametophyte (microgametophyte), while the ovules produce the female gametophyte (megagametophyte or embryo sac). A meiotic division of specific precursors within reproductive organs determines the transition from the sporophytic to the gametophytic phase. In the ovary, the ovule usually contains a single somatic cell (the megaspore mother cell or MMC) that undergoes meiosis to give rise to four haploid cells, so-called megaspores. In many flowering plants (including *Arabidopsis thaliana*, *Oryza sativa*, *Carica papaya* and *Zea mays*), a single megaspore is the precursor of the female gametophyte, as the other three meiotically derived cells degenerate soon after meiosis. The formation of the differentiated female gametes contained within a gametophyte requires several mitotic divisions, occurring within a single conspicuous, multinucleated cell (a syncitium). A typical differentiated female gametophyte of the *Polygonum* type, the most commonly found among angiosperms, is composed of seven cells, namely two synergids, an egg cell, a binucleated central cell and three antipodals. After gamete differentiation and pollination, a double fertilization event is responsible for initiating seed formation, establishing the sporophytic generation and giving rise to both the embryo and the transient endosperm.

N. Sánchez-León and J.-P. Vielle-Calzada
National Laboratory of Genomics for Biodiversity, Cinvestav Campus Guanajuato, Km 9.6 Libramiento Norte Carretera Irapuato-Leon, CP36500, Irapuato Guanajato, Mexico
e-mail: vielle@ira.cinvestav.mx

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This chapter reviews recent advances in the genetic and molecular understanding of female gametophyte development and function, emphasizing results associated with global approaches that allowed large-scale transcriptional analysis in *A. thaliana*. Based on these recent advances and their biological interpretation, crucial questions and problems are also discussed that remain to be solved in the coming years.

11.2 The Formation of Female Gametes

The female reproductive organ of angiosperms is called the gynoecium or, more commonly, the pistil. It is usually located at the centre of the flower. It is composed of three distinct tissues, namely the stigma, style and ovary. The stigma, located at the aerial distal extremity, is responsible for pollen grain reception, adherence and pollen germination. The style is a longitudinal tissue that separates the stigma from the ovarian cavity and is necessary to conduct growing pollen tubes towards the ovules. The ovary is composed of one or several cavities (locules) that harbour the ovules. An ovule consists of one or two integuments enclosing a group of inner cells that form the nucellus. The formation of the female gametophyte occurs within the nucellar tissue.

11.2.1 Megasporogenesis

Female gametogenesis is divided into two sequential phases, megasporogenesis and megagametogenesis, occurring during ovule formation. In most flowering species examined to date, primordial ovules arise from periclinal divisions of the inner ovary wall of the young gynoecia and extend into short finger-like projections, which are the origin of the integuments and delineate the nucellus (Bouman 1984). The establishment of the gametophytic phase requires the specification and differentiation of gamete precursors. During megasporogenesis, a single somatic cell in the young ovule primordium differentiates into a megaspore mother cell (MMC), the precursor of the meiotic products (Fig. 11.1). To date, the molecular mechanisms that control the specification of a single meiotic precursor are not clear. SPOROCYTELESS, a nuclear protein presumed to act as a MADS-like transcription factor, has been shown to be required for the differentiation of both male and female meiotic precursors (Yang et al. 1999). In other species that show sexual and apomictic reproduction, such as *Pennisetum ciliare*, a member of the Poaceae, multiple subepidermal cells of the ovule primordium enlarge and differentiate in the meiotic precursors. However, a single MMC is formed as the neighbouring cells are reabsorbed by the enlargement of the MMC undergoing meiosis. The differentiation of multiple MMCs in several species suggests that at least a cluster of somatic cells in the ovule primordium has the potential to enter meiosis.

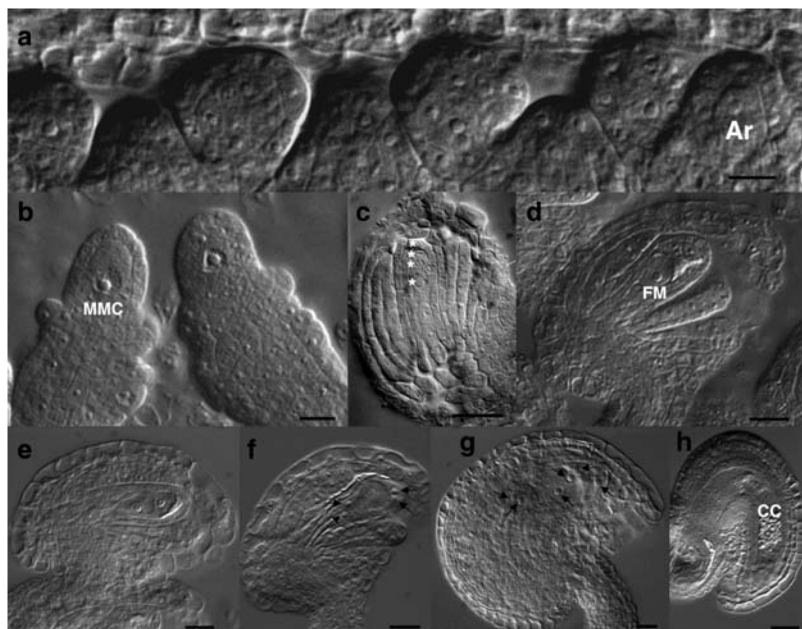

Fig. 11.1 Female gametophyte development in *Arabidopsis thaliana*. All micrographs represent whole-mount cleared ovules observed under Nomarsky optics. **a** Young ovule primordia at the onset of meiotic precursor differentiation. Primordia are in either a longitudinal or a perpendicular orientation with respect to the focal plane; a young archeosporal (Ar) cell is visible. Scale bar = 5 μm. **b** Ovule primordial containing megaspore mother cell (MMC) prior to meiosis. Scale bar = 10 μm. **c** Developing ovule at the end of megasporogenesis; all four meiotically derived megaspores are visible (*asterisks*); one megaspore is already degenerated. Scale bar = 10 μm. **d** Ovule containing a functional megaspore (FM) prior to megagametogenesis. Scale bar = 10 μm. **e** Ovule containing a female gametophyte at the 2-nucleate stage. Scale bar = 10 μm. **f** Ovule containing a female gametophyte at the 4-nucleate stage (*black arrows*). Scale bar = 10 μm. **g** Ovule containing a female gametophyte at the 8-nucleate stage; five nuclei are visible in this plane of focus (*black arrows*). Scale bar = 10 μm. **h** Ovule containing a fully differentiated female gametophyte with abundant presence of amylosplast in the central cell (CC). Scale bar = 10 μm

The specification and commitment of these cells is likely to be controlled by repressive mechanisms that have yet to be determined.

In most of the cereals (including maize), cell wall formation occurs immediately following meiosis I. However, in *Arabidopsis thaliana*, meiosis occurs without cytokinesis and it is only at the end of meiosis II that a cell wall isolates individual meiotic products. In contrast to male gametogenesis, where many haploid precursors differentiate simultaneously within the anther, the MMC is the only cell that undergoes meiosis within the ovule primordium, giving rise to a tetrad of haploid megaspores. In more than 70% of species examined cytologically, three meiotically derived cells invariably die. Only the innermost megaspore, deeply embedded in the ovule, gives rise to the functional megaspore, which is the only haploid precursor of the female gametophyte.

11.2.2 Megagametogenesis

The nucleus of this single meiotic product undergoes three consecutive rounds of mitosis without cytokinesis to form a voluminous syncitium with eight haploid nuclei. After mitotic arrest, cellularization and differentiation occur within the syncitium (Fig. 11.1). Most of the space is occupied by a binucleated central cell. At the distal pole, two synergids and the egg cell give rise to the egg apparatus, while at the proximal pole, three antipodals complement the seven cells of the most common female gametophyte found in flowering plants. These cells have defined positions within the embryo sac. It is believed that cell positioning, intracellular polarity and intercellular signalling are key determinants of cell specification, probably through the local action of hormones (Gross-Hardt et al. 2007). While most plant species follow this “monosporic” type of megagametogenesis, other species with either two (bisporic) or all four (tetrasporic) meiotic products survive and participate in female gametogenesis, contributing several cells to the final organization. Interestingly, the tetrasporic type of development gives rise to a heterozygous female gametophyte, in which haploid cells can carry distinct allelic contributions, an attribute yet to be exploited genetically to investigate haploid interactions among female gametophytic cells.

In Arabidopsis, but not in maize, the antipodal cells that are located at the most chalazal end of the female gametophyte degenerate prior to fertilization. Although these cells may be involved in the import of maternal nutrients to the fertilized embryo sac, their specific reproductive function has yet to be determined (Diboll 1968; Grossniklaus and Schneitz 1998). The absence of antipodals in female gametophytes of many species suggests that their function is not essential for sexual reproduction. However, detailed ultrastructural studies have shown large membrane invaginations that suggest an important nutritional exchange between the antipodals and the rest of the female gametophyte (Huang and Sheridan 1994). The antipodal cells are, together with the synergids, two lineages of accessory cells. The two synergids are located at the most micropylar end of the female gametophyte. Unlike antipodals, synergids are essential for fertilization, since they are necessary for the attraction of the pollen tube and the release of the sperm cells (Higashiyama et al. 2001; Huck et al. 2003; Rotman et al. 2003). The two lineages of true reproductive cells are the egg cell and central cell. The egg cell is located adjacent to the synergids and is highly polarized, with a large vacuole at the chalazal end of the cell, and the nucleus and most of the cytoplasm at the micropylar pole, as in maize, rice and Arabidopsis (Jensen 1965; Schultz and Jensen 1968; Cass et al. 1985; Sumner and van Caeseele 1989). This position favours the process of double fertilization by reducing the distance travelled by the sperm nucleus within the egg cell cytoplasm. The conspicuous central cell is located in the centre of the female gametophyte and is characterized by the presence of a large vacuole and many cytoplasmic organelles. It contains two nuclei that, in the case of Arabidopsis, fuse prior to fertilization with the sperm cell.

At maturity, the integuments completely enclose the nucellus and the differentiated female gametophyte, leaving a small physical aperture, the micropyle, through which the pollen tube is able to grow before penetrating a degenerated synergid and releasing the two sperm cells.

11.3 Genetic Dissection of Female Gametogenesis

Amongst the 29,000 predicted open reading frames that have been identified in Arabidopsis, it is believed that only a few thousand genes could be involved in the development of the female gametophyte (Drews and Yadegari 2002; García Hernández et al. 2002). Gametophytic mutations that affect either the male or female haploid generation exhibit non-Mendelian segregation and are not transmitted through the gametes. In the case of mutations that affect the female gametophytic phase, the defect in principle can be transmitted only to the next generation by a sperm cell of heterozygous individuals, because defective female gametophytes that carry a mutant allele are not viable. However, in practice, most female gametophytic mutations are not fully penetrant and, therefore, allow the recovery of homozygous individuals at low frequency.

The exact developmental stage in which the ovule initiates the gametophytic phase has not been determined to date. It is generally agreed that, since female meiosis occurs in a syncitium, sporophytic mutations that occur at the diploid level are likely to control MMC specification and differentiation, meiosis, post-meiotic cytokinesis and all aspects of female gametogenesis that are dependent on the surrounding sporophytic tissue. In contrast, gametophytic mutations have been commonly detected when these mutations affect the developmental aspects of the plant following meiosis. These include megagametogenesis, progamic functions of the female gametophyte (pollen tube guidance and double fertilization) and mechanisms that exert a maternal control over early seed formation. To date, several hundred gametophytic mutants have been analyzed that affect female gametogenesis in Arabidopsis. These mutants have been used to identify hundred of genes that are required for female gametogenesis or early seed development, by imposing a maternal effect following double fertilization (Moore et al. 1997; Christensen et al. 1998, 2002; Guitton et al. 2004; Pagnussat et al. 2005). Most mutants have been shown to possess defective female gametophytes impaired at key developmental stages during gametogenesis (Yadegari and Drews 2004), with a greater proportion either arrested before the first haploid mitosis, or defective in functional mechanisms occurring after cellularization (Pagnussat et al. 2005). Genes that have been characterized in detail encode several cell cycle proteins, including *PROLIFERA*, *ANAPHASE PROMOTING COMPLEX-2*, *NOMEGA* and *RETINOBLATOMA RELATED-1* (Springer et al. 1995; Kwee and Sundaresan 2003; Capron et al. 2003; Ebel et al. 2004), transcription factors, such as *MYB98* or *AGAMOUS-LIKE-80* (Kasahara et al. 2005; Portereiko et al. 2006), chromatin remodelling proteins acting either before (*CHR11*; Huanca-Mamani et al. 2005) or

after fertilization (members of the *FERTILIZATION INDEPENDENT SEED* class and their derivatives), controlling cell proliferation in both the embryo and the endosperm (Ohad et al. 1996; Chaudhury et al. 1997; Grossniklaus et al. 1998; Guitton and Berger 2005), and putative signalling proteins important for female gametophytic progression (*AGP18*, Acosta-García and Vielle-Calzada 2004; *CYTOKININ INDEPENDENT-1*, Pischke et al. 2002). Other genes that have been functionally characterized include the WD40 protein involved in haploid mitotic progression (*SLOW WALKER-1*; Shi et al. 2005), mitochondrial protein required for degeneration of the synergids (*GAMETOPHYTIC FACTOR-2*; Christensen et al. 2002) and lysophosphatidyl acyltransferase that is localized in the endoplasmic reticulum and is essential for female, but not male, gametogenesis (*LPAT-2*; Kim et al. 2005).

Recently, the mechanisms that underlie the formation of female gametophytic cells have started to be elucidated. In the *lachesis* (*lis*), *clotho* (*clo*) and *atropos* (*atos*) mutants, supernumerary egg cells differentiate at the expense of synergids, indicating that *LIS*, *CLO* and *ATOS* participate in cell-fate decisions within the female gametophyte (Gross-Hardt et al. 2007; Moll et al. 2008). *LIS*, *CLO* and *ATOS* encode a protein closely related to either yeast or human splicing factors, showing for the first time the importance of RNA editing in the determination of female gametophytic cells. In *eostre*, cell-fate switch of synergid to egg cell is also affected, in addition to haploid nuclear migration abnormalities. The *eostre* phenotype is caused by misexpression of the homeotic gene *BEL-LIKE HOMEODOMAIN-1* (*BLH1*); the protein is likely to form a heterodimer with KNAT3 (Pagnussat et al. 2007). It was suggested that this complex could be regulated by Ovate family proteins (OFPs) in Arabidopsis. A recent breakthrough elegantly presented by Pagnussat et al. (2009) has demonstrated that an auxin gradient established at early developmental stages directs cell identity in the differentiated female gametophyte. The acquisition of a specific cell identity depends on the distance that separates uncellularized nuclei from the micropylar end. These results indicate that auxin is an important morphogen acting in the female gametophytic phase of the life cycle.

11.4 Transcriptional Analysis of the Female Gametophyte

For many years, the small size of the Arabidopsis ovule has impeded the implementation of reliable techniques for the isolation of large amounts of gametophytic cells. Recently, a combination of genetic strategies and technologies for large-scale transcriptional analysis resulted in the identification of a significant number of genes that are expressed in the female gametophyte. The comparison of transcripts present in wild-type ovules and mutant ovules lacking a fully differentiated female gametophyte appears to be a powerful approach to distinguish genes that are active in the female gametophyte.

11.4.1 Gene Expression in the Differentiated Female Gametophyte

Using a highly penetrant *sporocyteless* (*spl*) allele in which 97% of female gametophytes are replaced by sporophytic cells of nucellar appearance, Yu et al. (2005) conducted ATH1 microarray experiments to compare the transcripts at two developmental stages of ovule formation, i.e. ovules undergoing megagametogenesis and fully differentiated ovules. The absence of transcriptional activity in *spl* suggests that the corresponding gene is either expressed specifically in cells of the female gametophyte, or negatively regulated by the activity of *SPOROCYTELESS*. Although the results did not reveal the difference between these two possibilities, 202 genes were identified that might be expressed in the female gametophyte, but not the rest of the ovule. Furthermore, five of these genes identified were later confirmed to be gametophytic-specific. A similar approach was used to identify genes with reduced expression in ovules of *determinant infertile1* (*dif1*; Steffen et al. 2007), a meiotic recessive sporophytic mutant in which 75% of fully differentiated ovules lacked the female gametophyte and 25% contained a one-nucleated cell reminiscent of the MMC. Steffen et al. (2007) hybridized the ovule RNA probes with ATH1 genome arrays and validated candidates using real-time reverse transcriptase PCR (RT-PCR). Amongst the 71 genes confirmed as differentially expressed by RT-PCR, 43 genes were validated using transgenic plants transformed with a translational promoter::GFP fusion. These genes were shown to be expressed constitutively in the female gametophyte or in a subgroup of cells (predominantly in the central cell, exclusively in the antipodals or predominantly in the synergids). In addition, one gene (*At2g21740*) was detected that was expressed exclusively in the egg cell, but its biological function is unknown.

Johnston et al. (2007) reported the use of genetic subtraction and microarray-based differential expression profiling to identify candidate genes expressed in the female gametophyte. In this study, fully differentiated gynoecia were employed. *Coatlique* (or *coatlicue*, meaning “the one with the skirt of serpents” and representing the goddess of war in Aztec mythology; *coa*) is a recessive sporophytic mutant that affects early megagametogenesis. In *coa*, 85% of differentiated ovules lack a female gametophyte but contain a single conspicuous haploid cell corresponding to the functional megaspore (Perez-Ruíz and Vielle-Calzada, unpublished data). Johnston et al. (2007) also conducted statistical analyses based on the results reported in a previous study (Yu et al. 2005). The differentially expressed candidate genes were identified by subtracting the transcriptomes of *coa* and *spl* from the wild type. By examining the datasets of previously reported embryo sac-expressed genes, the value of the fold-change was shown to reduce considerably, setting the baseline cut-off for subtraction at 1.28-fold in the wild type (compared to the usual twofold change). With this value, a total of 1,260 genes were identified as candidates for expression in the female gametophyte.

However, a twofold change in baseline was used to identify genes that showed sporophytic expression in *coa* or *spl* mutant ovules, because sporophytic cells in the ovule are abundant and their mRNA constitutes the majority of the universe transcripts that are detected. In addition, 12 new mutants showing either gametophytic or zygotic lethality were identified using in situ hybridization (Johnston et al. 2007).

Jones-Rhoades et al. (2007) compared the transcriptional activity in ovules between the wild-type *Columbia* and *dif1* and *myb98* mutants using whole-genome tilling microarrays. The *myb98* mutant has been characterized by impaired pollen tube attraction. The probes used were considered differentially expressed if $p \leq 0.05$ and $\log_2$ fold-change≥1. An interval showing differential expression was selected only if at least three neighbouring probes within 80 bp of each other showed the above threshold value. A total of 1,099 intervals were down-regulated in *dif1* ovules compared to wild type. While 969 intervals were shown to map to known annotated genes, 22 overlapped with ovule cDNA alignments reported as new coding sequences. The results of the *dif1* experiments have led to the identification of 382 protein-coding gene candidates expressed in the female gametophyte. The majority was predicted to encode putatively secreted proteins (78%) of mostly low molecular weight (<20 kDa) belonging to the multigene families. Less than 50% of the genes were represented in the classic ATH1 microarray that has been used in previous studies. A total of 59 genes were validated using RT-PCR, focusing on members of the gene family *Domain of Unknown Function* 784 group (*DUF784*), in which many members require *MYB98* for expression in the female gametophyte. These findings have led to the discovery of many paralogs that are expressed specifically in the ovule. Also, it may be functionally redundant in the female gametophyte of Arabidopsis, with the possibility that their functions have not been identified by forward genetic screens on the basis of loss-of-function mutations. Furthermore, transcriptional analysis of *myb98* ovules resulted in the identification of 77 down-regulated genes, amongst which 76 were also down-regulated in *dif1* ovules. These results suggest a collection of genes expressed in the female gametophyte, and that these genes might be involved in pollen tube guidance.

Several studies discussed above relied on microarray-based global expression analysis, which has shown a limited overlap in these datasets (Fig. 11.2). This may be explained by the use of *spl*, *dif1* and *coa* mutants, in which down-regulated transcripts are detected. While each of these mutants has been characterized by a high percentage of ovules lacking a female gametophyte, gametophytic cell fate in these mutants differs as their nucellus contains distinct cellular structures that require further developmental characterization. There has been no attempt to characterize cell identity in these defective organs. Therefore, it is likely that some of the differences observed may be due to distinct developmental programs that result in down-regulation of non-equivalent transcript collections, including those corresponding to genes that are not expressed in the female gametophyte, but repressed by the absence of *SPL*, *DIF1* or *COA* activity in the ovule. To date, genetic interactions between these three loci have not been

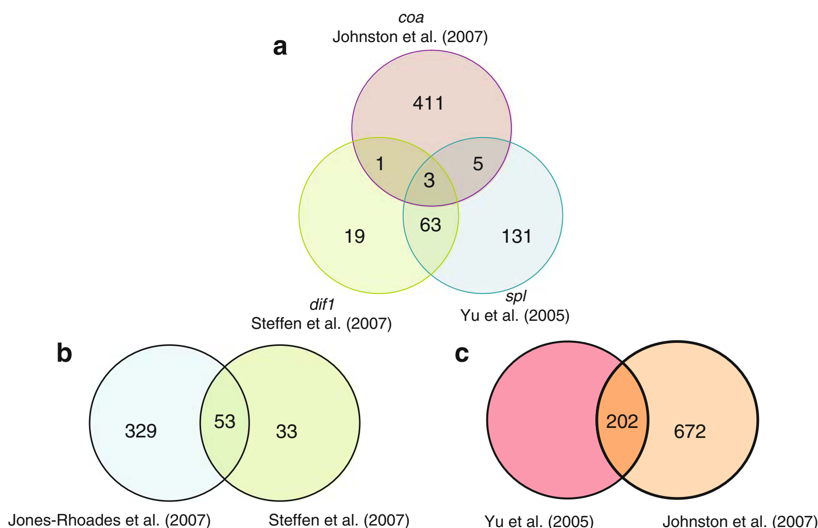

Fig. 11.2 Microarray experimental design and statistical analysis significantly affect the universe of transcripts presumably expressed in the female gametophyte. **a** Distribution of female gametophyte-specific gene candidates in similar experiments conducted with three distinct mutant backgrounds. **b** Distribution of female gametophyte-specific gene candidates in the identical mutant background (*dif1*) but using a different platform (ATH1 or tilling microarray). **c** Distribution of female gametophyte-specific gene candidates on the basis of two different statistical analyses of the same experimental results

investigated. If forward genetic analysis suggests that the female gametophyte transcriptome is composed of several thousands of genes (Drews and Yadegari 2002), then it is possible that these experiments have led to the identification of different subsets of non-overlapping genes. The sensitivity of different experiments is probably similar, if not identical. Therefore, other technologies that rely on large-scale transcript sequencing may represent an ideal complement to these strategies. The statistical analysis of each of these studies also differs, and this may contribute to discrepancies in their results. For example, a modified analysis of the data of Yu et al. (2005) generated a different collection of gene candidates according to Johnston et al. (2007), indicating that the overall results of these studies are prone to be corrected or modified in the coming years. Nevertheless, statistical analysis alone cannot ensure that genes down-regulated in mutant ovules are transcribed in the female gametophyte. The lack of transcriptional gene activity in mutant ovules may also be due to (1) female gametophyte-dependent gene activity in sporophytic cells of the ovule, (2) reduced expression in cells of the ovule sporophytic tissue, compared to expression of the same gene in the female gametophyte, and (3) a reduction in gene activity within mutant ovules, resulting in a decrease in transcription below the level of detection using microarray analysis.

11.4.2 Transcriptional Repression in Sporophytic Cells of the Ovule

Several genes have been identified that are overexpressed in mutant ovules lacking the female gametophyte. Johnston et al. (2007) reported the identification of 527 genes overexpressed in ovules of *spl* or *coa*, while 35 genes in *dif1* ovules have been shown to be up-regulated (Jones-Rhoades et al. 2007). Although many of these genes could be up-regulated in the absence of *SPL*, *DIF1* or *COA* activity, they could also represent genes that are repressed by the presence of the female gametophyte in wild-type ovules. To date, the genetic basis and molecular mechanisms that are involved in the interaction between sporophyte and gametophyte in the ovule are not clear, but it is plausible that specific mechanisms of genetic control are required for growth of the female gametophyte within the nucellus. Johnston et al. (2007) detected the overexpression of five genes in ovules of *coa* by in situ hybridization. These genes include *AT4G12410* (a *SAUR* (auxin-responsive Small Auxin Up RNA) gene), *AT1G75580* (an auxin-responsive gene), *AT5G03200* (encoding C3HC4-type RING finger protein), *AT5G15980* (encoding PPR repeat-containing protein) and *STM* (a homeo box gene). All genes have been shown to express strongly in the carpel wall and septum, compared to the sporophytic tissues of the ovule. Since fully differentiated gynoecia were used in these studies, additional large-scale transcriptional analysis using isolated ovules may lead to identification of genes that are repressed within the ovule sporophytic tissue in the presence of the female gametophyte.

11.5 Double Fertilization

Flowering plants are characterized by an original process of gamete fusion, in which two sperm cells fuse with an egg and central cell respectively (Nawashin 1898; Guignard 1899; Friedman 1990). The process precludes the transport of the sperm cells to the surface of the egg and central cell, and the fusion of male and female gametes (Weterings and Russell 2004). Although the findings of recent studies have shed light on the molecular and genetic control of pollen tube attraction and sperm release, knowledge has been limited regarding the subsequent steps of the fertilization process.

Synergids control some crucial mechanisms of pollen tube attraction within the ovule (Higashiyama et al. 2001). Individual cell laser ablation in *Torenia fournieri*, a member of the family Scrophulariaceae, showed that synergids are essential for guiding the pollen tube into the female gametophyte through the micropyle. The results of a later study indicated that diffusible pollen tube attractants produced by synergids might provide short-range localized signals that control pollen tube guidance (Higashiyama and Hamamura 2008). Recently, two cysteine-rich polypeptides (CRPs) related to defensin-like proteins, LURE1 and LURE2, were shown

to attract pollen tubes when secreted by the synergids in *T. fournieri* (Okuda et al. 2009). Interestingly, this attraction was not shared with other species, indicating that attractant specificity may be an unforeseen attribute of flowering plants. This preferential attraction is not in line with the results of earlier studies that suggested the possibility of calcium ions to serve as an universal attractant derived from synergid cells (Chaubal and Reger 1992). It is likely that CRPs are not the only peptides secreted by synergids acting as attractants, as other types of molecules having a major role in pollen tube guidance have also been identified. In maize, ZEA MAYS EGG APPARATUS1 (ZmEA1) is presumably a secreted small protein that is expressed abundantly in synergid cells (Márton et al. 2005). *ZmEA1* is a member of the EA1-like (EAL) gene family that possesses the EA box close to the C-terminal end (Gray-Mitsumune and Matton 2006). In Arabidopsis, *MYB98* encoding a R2R3-MYB transcription factor is also required for pollen tube guidance (Kasahara et al. 2005). The *myb98* mutants exhibit defective morphology of the filiform apparatus and are unable to attract pollen tubes. These defects could result from a reduced secretion of a putative pollen tube attractant (Punwani and Drews 2008). MYB98 has been shown to function as one of several transcriptional regulators that activate the expression of genes required for pollen tube guidance and the formation of the filiform apparatus (Punwani et al. 2007; Punwani and Drews 2008).

Genetic evidence from a recent study showed that *FERONIA*, encoding a receptor kinase expressed specifically in synergids, is essential for the arrest of pollen tube growth (Escobar-Restrepo et al. 2007). This finding indicates, for the first time, that cross-talk between female and male reproductive cells occurs before gamete fusion. In the *abstinence by mutual consent* (*amc*) mutant, pollen tube arrest is also prevented, but only when the *amc* pollen tube reaches a mutant *amc* female gametophyte. *AMC* encodes a peroxin that is associated with protein import into peroxisomes, indicating that an unknown diffusible molecule is required for pollen tube discharge (Boisson-Dernier et al. 2008). Additional genetic evidence confirms that sperm-specific genes are also important for pollen tube guidance. For example, *Generative Cell Specific 1* (*GCS1*) encodes a protein localized in the plasma membrane of male generative cells and sperm cells (Mori et al. 2006; von Besser et al. 2006; Márton and Dresselhaus 2008). This protein is likely anchored by a C-terminal transmembrane domain present in all members of the protein family. A second class of transmembrane proteins, represented by LGC1, has been reported in generative cells of *Lilium* (Xu et al. 1999), but its function in gamete recognition is not clear. Results from another study suggested a critical role of the central cell in pollen tube guidance, as observed in the central cell guidance mutant (*ccg*), which is defective in short-range pollen tube attraction (Chen et al. 2007). *CCG* encodes a nuclear protein that may play a role similar to that of TFIIB in yeast. Although the CCG expression pattern is weakly localized in the central cell cytoplasm, specific expression of *CCG* in the central cell rescues the mutant phenotype in heterozygous ccg/CCG individuals, indicating that the activity of the central cell is important for pollen tube guidance.

11.6 Future Trends

The alternation of generations is one of the most distinctive features of plants, with unicellular groups and related multicellular species exhibiting haploid/gametophyte-prevalent life cycles. Only vascular plants have evolved a dominant sporophytic multicellular stage that restricts the gametophytes to a small group of cells developing deep within specialized reproductive organs. The overall evolutionary tendency in most plant lineages has favoured a sporophyte-dominant life cycle reminiscent of the most prevalent multicellular eukaryotic lineages, including Basidiomycota (fungi) and Eumetozoa (animals). The ephemeral gametophytic phase of flowering plants substantially complicates the study of the genetic basis and molecular mechanisms that control female gametophyte development and function.

For the last two centuries, research on the plant life cycle has been largely descriptive, as plant biologists characterized morphologically a plethora of mechanisms and variants that occur during ovule and female gametophyte development. It is only in the last 20 years that the advent of model systems and large-scale sequencing technologies have begun to provide a complementary framework that could represent an initial step towards elucidation of the evolutionary basis of plant multicellularity and the alternation of generations. As projects, such as the Tree of Life, begin generating a phylogenetic landscape to approach the study of plant evolution on the basis of genomic information (Delwiche et al. 2004), large-scale efforts to characterize transcriptionally the ovule and female gametophyte could be of considerable importance to assemble the massive amount of data necessary to implement computational predictive analysis and modelling. In this regard, a rigorous assessment of the accuracy of transcriptional information would be crucial to confirm patterns of gene expression on the basis of experimental evidence. A comparative analysis of the first generation of microarray data suggests that a few hundred genes are found to be expressed in the female gametophyte. However, large variation in the collection of candidate transcripts that are identified by each of these experiments suggests that many could be the result of inaccuracies related to the technology. An interesting alternative that could complement these approaches relies on the use of large-scale sequencing technologies, namely Massively Parallel Signature Sequencing (MPSS; Peiffer et al. 2008) and its most recent derivative, Sequencing by Synthesis (SBS; Lister et al. 2008). Although these strategies are likely to have their own pitfalls, they promise to explore a different transcriptional universe and contribute to the overall representation of the female gametophyte transcriptome.

Although in-depth transcriptional mining can now be routinely conducted using a wide range of technologies, it is clear that experimental techniques that allow for the determination of in situ patterns of gene expression need improvement. Several new-generation vectors have facilitated the construction of translation fusions required for the testing of promoter activity (Curtis and Grossniklaus 2003), but the intricate molecular interactions that prevail at the sporophyte-gametophyte

cellular boundaries complicate the analysis. Our results comparing mRNA and protein localization during early megasporogenesis indicate that many genes show no equivalence between their site of transcription and their site of protein expression (Sánchez-León et al., unpublished data). Efforts to implement whole-mount in situ hybridization techniques in the ovule have yielded promising results (García-Aguilar et al. 2005), but now require a major effort to increase their scale through automation. Finally, the enhancement of in vivo image acquisition and analysis is confronted by major technological barriers that impede visual access during megasporogenesis and megagametogenesis. Although progress has been made in the analysis of pollen tube delivery in *Torenia* sp. and young seeds of Arabidopsis (Ingouff et al. 2007), the observation of living developing female gametophytes is a major challenge. The pioneering work of Olga Erdelska (1968, 1970) 40 years ago remains an important contribution that deserves attention and update. As plant biologists implement new strategies for developing a cohesive and articulated understanding of female gametogenesis in flowering plants, they have the opportunity to incorporate this exciting knowledge into the overall framework of biological mechanisms that shape the evolution of vascular plants.

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Chapter 12
Male Gametophyte Development

D. Twell

12.1 Introduction

The pollen grains produced by spermatophyte plants harbour haploid male germ cells that are vital for sexual reproduction. In flowering plants, their biological role is to nurture and deliver twin sperm cells, via the pollen tube, to the embryo sac for double fertilisation. Male gametophyte development has been the subject of intense investigation for more than a century and there exists an impressive literature concerning male gametophyte and gamete development, transport and fertilisation in a wide range of species. Apart from its intrinsic importance for sexual reproduction, pollen development represents a microcosm of cellular development in which to dissect the regulation of the fundamental processes of cell polarity, cell cycle regulation and gene expression, and how these are integrated with cell specification. Fuelled by advanced resources in genetic and genomic technologies, molecular and genetic research in pollen development has made substantial progress. The scope of this chapter concerns advances in our understanding of pollen development from the formation of unicellular microspores up to the point of pollen shed, based largely on progress achieved using genetic and transcriptomic approaches in *Arabidopsis* as a model. For other recent reviews, the reader is referred to McCormick (2004), Boavida et al. (2005), Honys et al. (2006a, b), Twell et al. (2006), Becker and Feijó (2007) and Singh and Bhalla (2007). Other chapters in this volume provide more detail on later events concerning pollen function, including pollen tube growth (Chap. 13) and fertilisation (Chap. 14). The reader is also referred to other recent reviews that discuss aspects of male fertility and pollen development that involve diploid cells of the sporophyte, including anther differentiation, male meiosis and the influence of the tapetum on pollen wall patterning (Wilson and Yang 2004; Scott et al. 2004; Ma 2005).

D. Twell
Department of Biology, University of Leicester, Leicester LE1 7RH, UK
e-mail: twe@le.ac.uk

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12.2 Overview of Pollen Development

In essence, pollen development reflects the ontogeny of a single cell from microspore inception to pollen release (Fig. 12.1). Pollen development begins in the anther locules and consists of two major phases, microsporogenesis and microgametogenesis. During microsporogenesis, the primary sporogenous layer gives rise

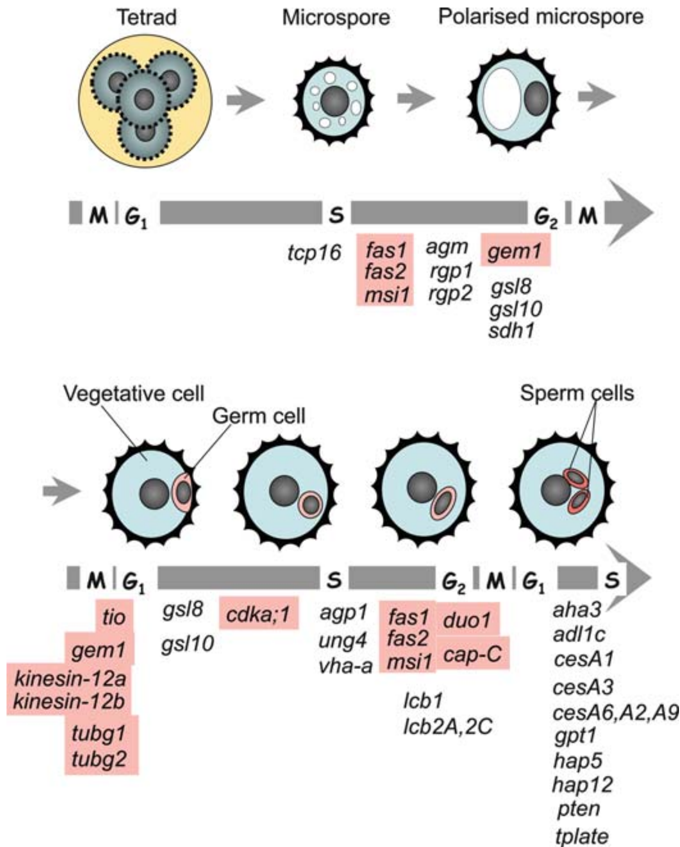

Fig. 12.1 Genes involved in male gametophyte development in *Arabidopsis*. Following male meiosis, a tetrad of haploid microspores is produced surrounded by a thick callose wall (*yellow*; note that all colour description refers only to the online publication of this chapter). Individual microspores released by dissolution of the callose wall undergo two mitotic divisions to produce mature tricellular pollen grains. The first asymmetric division gives rise to a vegetative cell (*blue*) that will form the pollen tube, and a smaller male germ cell (*pink*) that divides within the vegetative cell cytoplasm to form twin sperm cells (*red*). Stages in cell cycle progression in microspores and the male germ lineage are illustrated *below*. Genes with demonstrated roles at different stages of male gametophyte development are positioned on the developmental pathway according to their described mutant phenotypes. Mutants with direct effects on cell division and cell cycle-related processes are highlighted in *pink*

to the microsporocytes. Meiotic division of the diploid microsporocytes produces tetrads of haploid microspores enclosed within thick callosic walls, which are then released as individual microspores by an enzyme complex (callase) secreted by the tapetum (Scott et al. 2004). During microgametogenesis, microspores first undergo substantial cell growth that is associated with cell wall synthesis and vacuole biogenesis and fusion events (Owen and Makaroff 1995; Yamamoto et al. 2003). In parallel, the microspore nucleus migrates to the cell wall. These polarised microspores then undergo a highly asymmetric division at pollen mitosis I (PMI) giving rise to bicellular pollen grains.

Pollen mitosis I is a landmark event in pollen development that gives rise to daughter cells with different structures and fates (Twell et al. 1998). The large vegetative cell has dispersed nuclear chromatin and exits the cell cycle in G_1, while the smaller generative or male germ cell has condensed nuclear chromatin and divides once to form two sperm cells. After pollen mitosis, the germ cell is completely engulfed by the vegetative cell. Phagocytosis of the germ cell involves degradation of the hemispherical callose wall that separates the nascent vegetative and germ cells; the internalized germ cell then forms an elongated shape that is maintained by a cortical cage of bundled microtubules (Palevitz and Cresti 1989). Germ cell division at pollen mitosis II (PMII) takes place within a membrane bound compartment of the vegetative cell cytoplasm and a physical association is established between the germ cells and the vegetative nucleus, known as the male germ unit. The male germ unit is thought to be important for the coordinated delivery of the gametes and sperm cell fusion events (Dumas et al. 1998; Lalanne and Twell 2002). During pollen maturation the vegetative cell accumulates carbohydrate and/or lipid reserves required for the demands of plasma membrane and pollen tube wall synthesis (Pacini 1996). Moreover, pollen grains are finally dehydrated when released from the anthers and accumulated osmoprotectants, including disaccharides and proline or glycine-betaine, are thought to protect vital membranes and proteins from damage during dehydration (Schwacke et al.1999).

12.3 Gametophytic Mutants Affecting Pollen Development

Several genetic screening strategies have been applied to isolate gametophytic mutants and genes influencing pollen development and cellular functions in *Arabidopsis*. These include gametophyte-targeted forward genetic screens, gametophyte-directed reverse genetic screens and non-gametophyte-directed reverse genetic screens. Forward genetic strategies have proven effective in identifying developmental male gametophytic mutants. First, by morphological screening of pollen from mutagenized plants using histochemical staining for DNA (Chen and McCormick 1996; Park et al. 1998) or callose (Johnson and McCormick 2001) and, second, by analysis of pollen phenotypes in plants showing marker segregation ratio distortion (Bonhomme et al. 1998; Howden et al. 1998; Grini et al. 1999; Procissi et al. 2001; Johnson et al. 2004; Lalanne et al. 2004a, b). A recent survey

Table 12.1 Arabidopsis genes required during male gametophyte development. Genes with redundant functions are indicated with the same superscript letter a–e

Gene ID	Gene	Mutant	Mutant phenotype	Protein identity	Protein function	Reference
At1g14830	*ADL1C*	*arabidopsis dynamin-like 1C*	Pollen abortion during maturation	Dynamin-like protein/ renamed DRP1C	Pollen plasma membrane maintenance, intine synthesis	Kang et al. (2003)
At5g44860	*AGM*	*abnormal gametophytes*	Pollen degenerates at late microspore stage	Putative transmembrane protein	Microspore development	Sorensen et al. (2004)
At1g24520	*AGP1*	*arabinogalactan protein1 [AtBCP1]*	Pollen aborts and collapsed at bicellular stage	Arabinogalactan protein	Tapetum, microspore and bicellular pollen viability	Xu et al. (1995)
At5g57350	*AHA3*	*Arabidopsis H^+-ATPase 3*	Collapsed and aborted mature pollen	Plasma membrane H^+-ATPase	Nutrient transport, microspore/pollen maturation	Robertson et al. (2004)
At1g65470	*FAS1*	*fasciata1*	Microspore and male germ cell cycle arrest at G2/M	Chromatin Assembly Factor-1 (CAF-1) p150 subunit	Nucleosome/chromatin assembly during replication	Chen et al. (2008)
At5g64630	*FAS2*	*fasciata2*	Microspore and male germ cell cycle arrest at G2/M	Chromatin Assembly Factor-1 (CAF-1) p60 subunit	Nucleosome/chromatin assembly during replication	Chen et al. (2008)
At5g48600	*CAP-C*	*cap-C*	Chromosome bridges at PMII and in mature pollen	Catalytic subunit of SMC4 condensin complex	Chromatin condensation and segregation	Siddiqui et al. (2006)
At3g48750	*CDKA;1*	*cyclin-dependent kinase A;1*	Bicellular pollen: germ cell S-phase progression inhibited	Cyclin-dependent kinase A	Germ cell S-phase progression	Iwakawa et al. 2006, Nowack et al. 2006
At4g32410	*CESA1*	*cellulose synthaseA1*	Aborted mature pollen, irregular cell wall synthesis	Cellulose synthase catalytic subunit A1	Cellulose synthesis, intine deposition	Persson et al. (2007)
At4g39350	*CESA2*[a]	*cellulose synthaseA6*	Aborted mature pollen, irregular cell wall synthesis	Cellulose synthaseA6 catalytic subunit (redundant with CESA6, CESA9)	Cellulose synthesis, intine deposition	Persson et al. (2007)
At5g05170	*CESA3*	*cellulose synthaseA3*	Aborted mature pollen, irregular cell wall synthesis	Cellulose synthaseA3 catalytic subunit	Cellulose synthesis, intine deposition	Persson et al. (2007)

At5g64740	*CESA6*[a]	*cellulose synthaseA6*	Aborted mature pollen, irregular cell wall synthesis	Cellulose synthaseA6 catalytic subunit (redundant with CESA2, CESA9)	Cellulose synthesis, intine deposition	Persson et al. (2007)
At2g21770	*CESA9*[a]	*cellulose synthaseA6*	Aborted mature pollen, irregular cell wall synthesis	Cellulose synthaseA6 catalytic subunit (redundant with CESA2, CESA6)	Cellulose synthesis, intine deposition	Persson et al. (2007)
At3g60460	*DUO1*	*duo pollen1*	Bicellular pollen: germ cell fails to enter mitosis	R2R3 MYB transcription factor (MYB125)	Regulator of male germ cell specification and G2/M transition	Durbarry et al. (2005), Rotman et al. (2005)
At2g35630	*GEM1*	*gemini pollen1*	Twin-celled and binucleate pollen: abnormal divisions at pollen mitosis I	MOR1/GEM1: homologous to chTOGp/XMAP215 family of microtubule associated proteins	Microspore polarity and cytokinesis through microtubule organisation	Park et al. (1998), Park and Twell (2001), Twell et al. (2002)
At5g54800	*GPT1*	*glucose-6-phosphate translocator1*	Reduced lipid bodies, vesicles and vacuoles; aborted pollen at tricellular stage	Glucose-6-phosphate translocator	Glc6P import for plastidic starch, and fatty acid biosynthesis and carbon for OPPP	Niewiadomski et al. (2005)
At2g36850	*GSL8*	*glucan synthase-like 8*	Failed and abnormal microspore division, callose deposition, failed germ cell phagocytosis, pollen abortion	Similar to callose (b1-3 glucan) synthase	Microspore division, phragmoplast callose synthesis, GC phagocytosis	Töller et al. (2008
At3g07160	*GSL10*	*glucan synthase-like 10*	Failed and abnormal microspore division, callose deposition, failed germ cell phagocytosis	Similar to callose (b1-3 glucan) synthase	Microspore division, phragmoplast callose synthesis, GC phagocytosis	Töller et al. (2008)
At1g30450	*HAP5*	*hapless5*	Aborted mature pollen	Cation-chloride cotransporter	Ion homeostasis during development	Johnson et al. (2004)
At4g36900	*HAP12*	*hapless12*	Aborted mature pollen	Contains AP2 domain (RAP2.10)	Regulator of pollen gene expression	Johnson et al. (2004)

(*continued*)

Table 12.1 (continued)

Gene ID	Gene	Mutant	Mutant phenotype	Protein identity	Protein function	Reference
At4g14150	*KINESIN-12A*[b]	*KINESIN-12A*	Microspores fail to complete pollen mitosis I with phragmoplast defects	Kinesin-12 family	Phragmoplast microtubule organisation	Lee et al. (2007)
At3g23670	*KINESIN-12B*[b]	*KINESIN-12B*	Microspores fail to complete pollen mitosis I with spindle defects	Kinesin-12 family	Phragmoplast microtubule organisation	Lee et al. (2007)
At4g36480	*LCB1*	*long-chain base1 (fbr11-2)*	Apoptotic cell death at bicellular stage, aborted pollen at tricellular stage	Sphingoid long-chain base1 subunit of serine palmitolyltransferase (SPT)	Sphingolipid synthesis, repression of PCD	Teng et al. (2008)
At3g48780	*LCB2A*[c]	*long-chain base2A*	Apoptotic cell death at bicellular stage, aborted pollen at tricellular stage	Sphingoid long-chain base2 subunit of serine palmitolyltransferase (SPT)	Sphingolipid synthesis, repression of PCD	Dietrich et al. (2008), Teng et al. (2008)
At5g23670	*LCB2C*[c]	*long-chain base2C*	Apoptotic cell death at bicellular stage, aborted pollen at tricellular stage	Sphingoid long-chain base2 subunit of serine palmitolyltransferase (SPT)	Sphingolipid synthesis, repression of PCD	Dietrich et al. (2008), Teng et al. (2008)
At5g58230	*MSI1*	*multicopy suppressor of IRA1*	Microspore and male germ cell cycle arrest at G2/M	Chromatin Assembly Factor-1 (CAF-1) p48 subunit/pRbAp48 homologue	Nucleosome/chromatin assembly during replication	Chen et al. (2008)
At5g39400	*PTEN*	*Arabidopsis phosphatase and tensin homologue*	Pollen aborts after pollen mitosis II	Phosphatase and tensin homologue [tyrosine/PIP3 phosphatase]	Pollen maturation	Gupta et al. (2002)
At3g02230	*RGP1*[d]	*reversibly glycosylated polypeptide 1*	Microspore vacuolated, bicellular stage pollen collapsed, intine defective	Reversibly glycosylated polypeptide 1	Polysaccharide biosynthesis, intine synthesis, vacuole integrity	Drakakaki et al. (2006)
At5g15650	*RGP2*[d]	*reversibly glycosylated polypeptide 2*	Microspore vacuolated, bicellular stage pollen collapsed, intine defective	Reversibly glycosylated polypeptide 2		Drakakaki et al. (2006)

At3g45150	*TCP16*	*tcp16*	Microspore nuclear DNA loss and abortion	bHLH protein, TCP PCF-subfamily	Regulator of microspore gene expression	Takeda et al. (2006)
At1g50230	*TIO*	*two-in-one*	Microspores fail to complete cytokinesis at PMI	Homologous to FUSED-kinases	Signalling role in cell plate/phragmoplast expansion	Oh et al. (2005)
At3g01780	*TPLATE*	*tplate*	Pollen aborts at tricellular stage with ectopic callose synthesis	Similar to adaptin_N domain of COP1	Vesicle trafficking, intine synthesis	van Damme et al. (2006)
At3g61650	*TUBG1*[e]	*gamma tubulin1*	Abnormal spindle organisation at PMI, low frequency binucleate pollen	Gamma tubulin	Microtubule nucleation, spindle architecture	Pastuglia et al. (2006)
At5g05620	*TUBG2*[e]	*gamma tubulin2*	Abnormal spindle organisation at PMI, low frequency binucleate pollen	Gamma tubulin	Microtubule nucleation, spindle architecture	Pastuglia et al. (2006)
At5g66760	*SDH1*	*succinate dehydrogenase*	Microspores abort before pollen mitosis I	Succinate dehydrogenase (mitochondrial complex II)	Mitochondrial electron transport chain and TCA cycle	León et al. (2007)
At2g34550	*UNG3*	*ungud3*	Pollen aborts at bicellular stage	GA-2-oxidase	Gibberellin catabolism	Lalanne et al. (2004a, b)
At1g78900	*VHA-A*	*vacuolar-ATPase V1 subunit A*	Pollen aborts at bicellular stage and later; swollen ER cisternae	Vacuolar-ATPase V1 subunit A	Golgi organisation, pH homeostasis, secondary active transport	Dettmer et al. (2005)

identified approximately 50 gametophytic mutants and 13 genes that affect post-meiotic male gametophyte development before pollen release (Twell et al. 2006). At the time of writing, this list extends to 37 genes, representing a significant increase in knowledge in the past two years. A compilation of genes and their associated mutant phenotypes is presented in Table 12.1.

In the most common mutant class, pollen degenerates and aborts at various developmental stages, while a second important class specifically affects cell division and patterning. Pollen abortive phenotypes could arise from various mechanisms. For example *abnormal gametophytes* (*agm*) encodes a putative transmembrane protein, *vha-A*, a subunit of the vacuolar H^{+}-ATPase, *hap5*, an AP2 domain transcription factor, and *hap12*, a cation-chloride transporter (Table 12.1). Interestingly, tagged sequences in the *ung3* mutant encode a GA-2-oxidase, suggesting a role for gibberellin in pollen development (Lalanne et al. 2004b), as well as its recently discovered role in pollen tube growth (Singh et al. 2002; Chhun et al. 2007). In an increasing number of cases, genes with essential gametophytic roles have been discovered by characterising loss of function phenotypes caused by knockout or knockdown mutations in genes of interest. This approach has led to the identification of a number of essential genes, e.g. *AtPTEN*, *ADL1C*, *AHA3* and *GPT1* (Table 12.1). Overall, proteins with a variety of functions have been defined, including those with roles in cell division, chromatin organisation, cell wall synthesis, energy and metabolism, pH regulation and ion transport, and other groups with roles in transcriptional regulation, hormone catabolism and intracellular signalling (Table 12.1). The subset of genes with essential roles in microspore or germ cell division are highlighted on Fig. 12.1 and discussed in more detail below.

12.4 Mutants Affecting Gametophytic Cell Divisions (Morphological Screens)

A central question regarding cellular patterning during pollen development is how asymmetric division is linked to specification of vegetative and germ cell lineages. Manipulation of cell division at PMI, in combination with a vegetative cell fate marker, revealed a role for division symmetry in germ cell specification. As a result, two general models were proposed to account for differential cell fate arising from asymmetric division at PMI (Eady et al. 1995). Both assume that vegetative cell fate is the default gametophyte pathway and provide alternative mechanisms to explain how vegetative cell-specific genes are repressed in the germ cells. In essence, gametophytic activators may be simply excluded from the presumptive germ cell domain, or hypothetical repressors at the germ cell-pole may block vegetative cell-specific gene expression (Twell et al. 1998). Recent data (discussed below) based on molecular analysis of male germline-specific gene expression and the analysis of mutants affecting germline cell division, have led to a more detailed model of the control of cell proliferation and cell specification that is required to pattern the male gametophyte.

12.5 Genes with Roles in Asymmetric Microspore Division

In the *sidecar pollen* (*scp*) mutant, microspores undergo symmetrical division, followed by asymmetric division of only one daughter cell to produce mature pollen with a supernumerary vegetative cell. Although SCP gene remains to be identified, the *scp* phenotype highlights the importance of coordinating the mitotic cell cycle with the expression of polarity (Chen and McCormick 1996). *gemini pollen1* (*gem1*) displays a range of microspore division phenotypes including equal, unequal and partial divisions (Park et al. 1998). In contrast to *scp*, symmetrical divisions in *gem1* do not occur precociously and neither daughter cell completes a further division (Fig. 12.2a, b). Cell fate analysis in *gem1* supports the role of cell size or nuclear/cytoplasmic ratio as a factor in determination of cell fate (Park et al. 1998; Park and Twell 2001). GEM1 is identical to MOR1 (Whittington et al. 2001) and is homologous to the MAP215 family of microtubule-associated proteins that stimulate plus-end microtubule growth. MOR1/GEM1 colocalises with interphase, spindle and phragmoplast microtubule arrays and plays a vital role in microspore polarity as well as cytokinesis (Twell et al. 2002). A recent analysis of mutations in

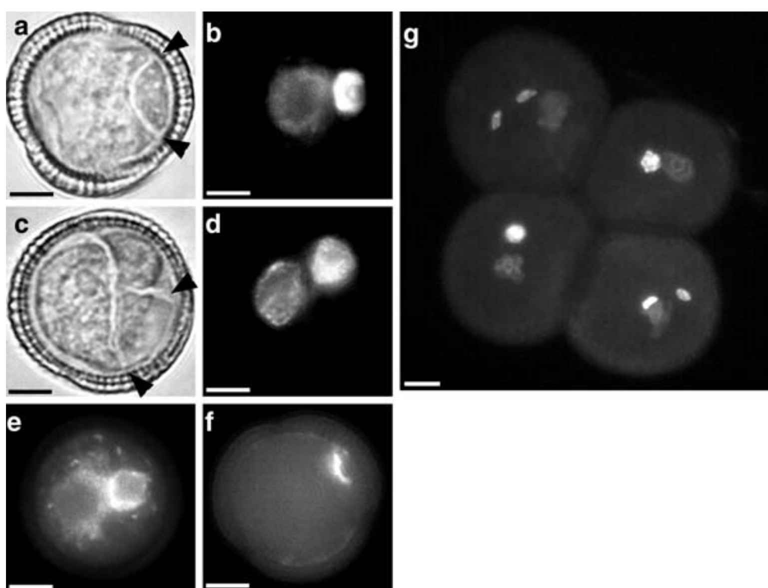

Fig. 12.2 Pollen phenotypes of selected *Arabidopsis* gametophytic mutants affecting cell division. **a**–**d** Bright field (**a**, **c**) and corresponding DAPI-stained (**b**, **d**) images of wild-type (**a**, **b**) and *gem1* (**c**, **d**) pollen at early bicellular stage. Wild-type pollen shows a typical hemispherical cell wall but *gem1* pollen grains often produce misplaced and branched internal cell walls. **e** DAPI-stained *tio* pollen with typical binucleate phenotype resulting from failed cytokinesis at pollen mitosis I. **f** *tio* pollen immediately after nuclear divison at PMI stained with aniline blue, showing an incomplete and transient callose cell plate. **g** Tetrad from heterozygous *duo1* mutant in the *quartet1* background showing 2:2 segregation for wild-type and *duo1* mutant pollen. Bars=5 μm

two gamma tubulin genes has revealed their redundant role in spindle organisation at PMI. Double mutants do not affect microspore polarity and result in disorganised spindles that lead to mitotic segregation defects (Pastuglia et al. 2006).

Asymmetric cytokinesis following PMI effectively isolates the germ cell and possesses two special features in that (1) no preprophase band of microtubules marks the future division plane, and (2) a unique transient curved cell plate is formed to enclose the germ cell nucleus. In the *two-in-one* (*tio*) mutant, microspores complete nuclear division, but fail to complete cytokinesis at PMI, resulting in binucleate pollen grains. In contrast to *gem1*, *tio* displays cytokinesis-specific defects resulting in cell plates that fail to expand (Fig. 12.2c, d). TIO encodes a plant homologue of the Ser/Thr protein kinase FUSED, a key component of the hedgehog signalling pathway in animals (Lum and Beachy 2004; Oh et al. 2005). TIO localises to the midline of the phragmoplast where it has an essential role in cell plate expansion. A redundant role has recently been discovered for the two KINESIN-12 family proteins, phragmoplast associated KINESIN-12A and KINESIN-12B. Double mutant mirospores complete chromosome segregation, but fail to assemble the characteristic antiparallel phragmoplast microtubule array, resulting in failure of cytokinesis at PMI (Lee et al. 2007).

12.6 Genes Controlling Male Germline Development

Several mutants have been isolated that disturb male germ cell cycle progression (Fig. 12.1). Components of the canonical cell cycle machinery are expected to play essential roles in microspore and germ cell division, but only recently has functional evidence emerged. Analysis of T-DNA insertion mutants in the single A type *CYCLIN-DEPENDENT KINASE* (*CDKA;1*) gene revealed an essential role in germ cell division (Iwakawa et al. 2006; Nowak et al. 2006). Although microspores are unaffected in *cdka;1* mutants, meiotic inheritance of CDKA;1 may prevent cell cycle defects. In *cdka;1* mutants, division of the germ cell fails. However, the resultant single germ cell is functional and exclusively fertilises the egg cell. This preferential fertilisation by mutant *cdka;1* germ cells may arise from positional constraints, signalling within the embryo sac, or involve incomplete gamete differentiation (Nowack et al. 2006). In the latter case, the expression of factors required for central cell fertilisation may be dependent on germ cell mitosis and/or CDKA;1 activity. Recent analysis of CHROMATIN ASSEMBLY FACTOR-1 (CAF-1) pathway mutants (*fas1*, *fas2*, *msi1*), in which pollen with a single germ cell indiscriminately fertilises either female gamete, is consistent with the hypothesis that *cdka;1* germ cells are incompletely differentiated. CAF-1 deficient pollen correctly expresses germ cell fate markers, suggesting that gamete cell fate is specified independent of germ cell cycle regulation (Chen et al. 2008). In contrast to *cdka;1* mutants, CAF-1 pathway mutants also disturb the microspore cell cycle, demonstrating a wider role in male gametophyte division that could involve direct

or epigenetic deregulation associated with nucleosome and chromatin assembly following replication (Chen et al. 2008).

duo pollen (*duo*) mutants complete asymmetric microspore division, but fail to undergo or complete germ cell division (Durbarry et al. 2005). Heterozygous *duo1* and *duo2* mutants produce ~50% bicellular pollen containing a single germ cell (Fig. 12.2e). *duo2* mutant germ cells enter mitosis but arrest at prometaphase, suggesting a specific role for DUO2 in mitotic progression of germ cells (Durbarry

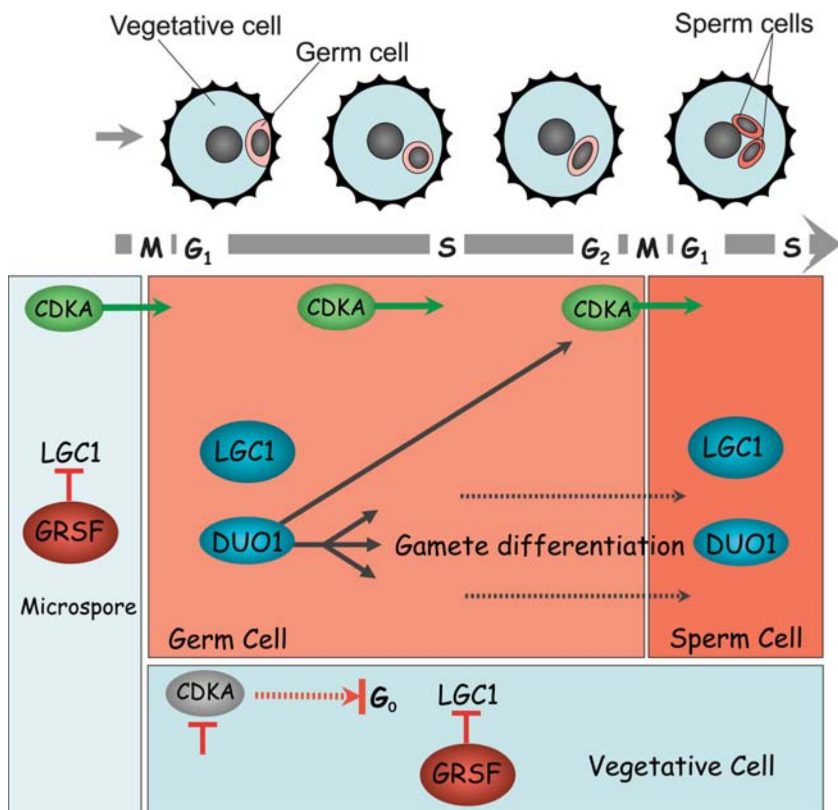

Fig. 12.3 Model of the role of positive and negative regulatory factors in male germline development. The repressor protein GRSF (GERMLINE RESTRICTIVE SILENCING FACTOR) is expressed in microspores and vegetative cells where it represses germline-specific genes such as *LGC1* (*LILY GENERATIVE CELL 1*) in lily. GRSF is excluded from the germline, thereby derepressing the *LGC1* gene. The positive regulator DUO1 (DUO POLLEN 1) is expressed exclusively in the nuclei of germline cells formed after asymmetric division of the microspore in *Arabidopsis*. DUO1 is required for the expression of genes involved in germline differentiation and G2/M phase progression that is dependent on activated CDKA (CYCLIN DEPENDENT KINASE A). Thus, the negative regulator GRSF is involved in germ cell specification by exclusion from the male germline, and the positive regulator DUO1 has an integrative role in promoting germ cell differentiation and division to form twin sperm cells. Different phases of the germline cell cycle are indicated as G_0, G_1, S, G_2 and M

et al. 2005). Mutant germ cells in *duo1* complete S-phase but fail to enter mitosis, suggesting that DUO1 may control the expression of G2/M phase regulators. DUO1 encodes a novel R2R3 MYB protein specifically expressed in germline cells, and DUO1 orthologues are present throughout the angiosperms (Rotman et al. 2005). Mutant *duo1* pollen grains contain a single germ cell that does not lead to successful fertilisation, suggesting that in addition to cell cycle defects, key features of gamete differentiation and function are incomplete. DUO1 may therefore act as a germ cell fate determinant linking cell division and gamete specification (Fig. 12.3).

In parallel to genetic studies, recent molecular analyses, including EST (expressed sequence tag) sequencing approaches, have provided compelling evidence for germline transcription in maize sperm cells and in lily generative cells (Engel et al. 2003; Okada et al. 2005). Several male germline-specific genes have also been characterised in *Arabidopsis* (Okada et al. 2005; Engel et al. 2005), including *GCS1*/*HAP2* that encodes a gamete surface protein required for pollen tube guidance and gamete fusion (Mori et al. 2006; von Besser et al. 2006). Male germ cell division can be uncoupled from gamete specification, as shown by analysis of CDK and CAF-1 pathway mutants (Iwakawa et al. 2006; Nowack et al. 2006; Chen et al. 2008). Moreover, a ubiquitous non-germ cell repressor protein, germline restrictive silencing factor (GRSF), has recently been identified in lily that represses the germline-specific LGC1 promoter in non-germ cells (Haerizadeh et al. 2006). This suggests that a transcriptional derepression mechanism is involved in male germ cell specification. Thus, a combination of negative and positive regulators of male germ cell-specific gene expression appears to be involved in regulating germ cell proliferation and specification. A schematic model incorporating data from lily and *Arabidopsis* is presented in Fig. 12.3.

12.7 Transcriptomics of Pollen Development

Characterisation of male gametophyte gene expression has reflected the available methodologies. This has included isozyme and RNA hybridisation studies that suggested an extensive pollen gene expression programme and a significant overlap of gametophytic and sporophytic gene expression. Moreover, until recently, gene-by-gene characterisation identified ~150 pollen-expressed genes from different species, with strong evidence for pollen-specific expression for about 30 (see Twell 2002). The availability of new high-throughput technologies has enabled analysis of the haploid male transcriptome on a global scale. Three initial studies, exploiting serial analysis of gene expression (SAGE) technology (Lee and Lee 2003) and 8K Affymetrix AG microarrays (Honys and Twell 2003; Becker et al. 2003), investigated the mature pollen transcriptome. Both approaches gave similar overall views of pollen gene expression, and the classification of pollen-expressed and pollen-specific genes into functional categories revealed several over-represented functional groups involved in cell wall metabolism, cytoskeleton and signalling among the pollen-specific genes.

Further refinement was enabled by the availability of Affymetrix 23K *Arabidopsis* ATH1 arrays in three key studies. The first generated microarray data covering four stages of male gametophyte development for ecotype Landsberg *erecta* (Honys and Twell 2004). Two further studies reported ATH1 datasets for mature pollen from ecotype Columbia (Zimmermann et al. 2004; Pina et al. 2005). Estimations of the number of genes active in the male gametophyte are dependent on data treatment, especially on microarray normalisation algorithms and the treatment of detection calls in replicates (Boavida et al. 2005; Twell et al. 2006; Becker and Feijó 2007). Taken together, it is estimated that the total number of genes expressed in mature pollen lies between 5,000 and 7,000. Considering all developmental stages, about 14,000 genes gave a positive expression signal during male gametophyte development (Honys and Twell 2004; Twell et al. 2006).

Comparative analyses of the male gametophytic transcriptomes can now be performed against an increasing number of sporophytic datasets, increasing their resolution. This has led to a decrease in the number of strictly male gametophyte-specific genes in a recent analysis (about 5% of all male gametophyte-expressed genes), compared with previous estimates of about 10% (Honys and Twell 2004; Pina et al. 2005). Moreover, male gametophyte-specific genes are often characterised by very high expression levels, highlighting their importance in the specialized gametophytic programme (Honys and Twell 2004, Honys et al. 2006a).

The impact of these studies lies in the massively increased knowledge of the complexity and dynamics of haploid gene expression (Honys and Twell 2004). To enable rapid assessment of developmental gene expression and comparison with selected sporophytic datasets, pollen transcriptomic datasets have recently been assembled in a normalized database that includes visualization tools—*Arabidopsis* Gene Family Profiler (aGFP; Dupl'ková et al. 2007).

12.8 Two Global Male Gametophytic Gene Expression Programmes

Male gametophyte gene expression can be divided into two major phases, early and late, which comprise microspore and bicellular pollen, and tricellular and mature pollen. However, there is significant overlap and the vast majority of genes are expressed in both phases. In individual stages, the number of active genes is maintained at approximately 11,000 in microspores and in bicellular pollen, but then declines progressively to about 7,000 in mature pollen (Fig. 12.4). Early profiles are significantly more similar to those of the sporophyte than to late pollen stages, which reflects their undifferentiated state and the potential inheritance of pre-meiotic transcripts in microspores and bicellular pollen (Honys and Twell 2004). The switch point between both developmental programmes occurs prior to PMII, and the absence of most abundant early male gametophyte-specific genes in tricellular pollen datasets clearly demonstrates this switch. Moreover, the majority

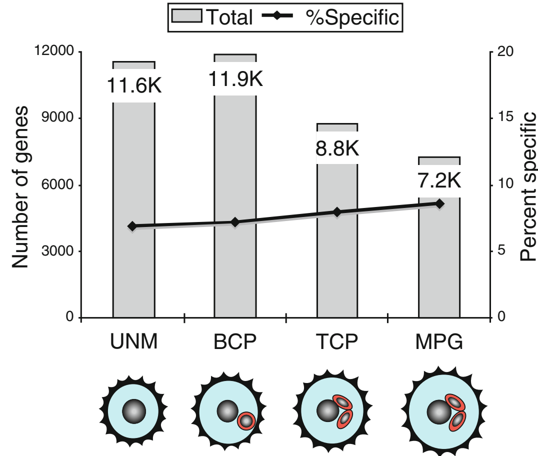

Fig. 12.4 Transcriptomic analysis of male gametophyte development. *Bars* show the total number of genes expressed in uninucleate microspores (UNM), bicellular pollen (BCP), tricellular pollen (TCP) and mature pollen grains (MPG). The percentage of male gametophyte-specific genes expressed at each developmental stage is plotted

of highly expressed late phase genes show pollen-specific expression patterns. This reduction in complexity and switch to the late genes enriched in categories involved in cell wall metabolism, cytoskeleton and signalling highlights the uniqueness of the late pollen transcriptome, and the functional specialization of pollen in preparation for maturation and rapid pollen tube growth (Honys and Twell 2003, 2004).

These overlapping global gene expression programmes demand coordinated regulation at the transcriptional level. Of approximately 1,350 predicted transcription factors in *Arabidopsis*, 612 were expressed in developing male gametophytes (542 early, 405 late). Of these, 76 were pollen-enhanced or pollen-specific and represent strong candidates in the regulatory networks that operate in male gametophyte development (Honys and Twell 2004). A recent pioneering study has combined transcriptomic and mutational analysis to establish a late pollen regulatory network. Analysis of transcriptomic data identified a subset of five MIKC* MADS box proteins (AGL30/65/66/94/104) that are specifically expressed during late stages of pollen development (Honys and Twell 2004; Pina et al. 2005). Double mutant combinations affect in vitro pollen germination and pollen fitness in planta (Verelst et al. 2007a). Transcriptomic analysis of pollen from single, double and triple mutant combinations of interacting pairs of MIKC* proteins allowed the dissection of putative target genes of the five pollen-specific MIKC* MADS box complexes (Verelst et al. 2007b). These include structural and regulatory genes such as two non-MIKC* MADS proteins (AGL18 and AGL29) that were identified as downstream regulators of a subset of MIKC* regulated genes. Further analysis of the architecture of this late network, together with ongoing mutational analysis of different classes of stage-specific pollen transcription factors, will allow more complex regulatory models to be established, including their interactions and their significance in pollen development and fitness.

12.9 Post-Transcriptional Regulation

Pollen germination in many species has been shown to be largely independent of transcription but vitally dependent on translation (see Twell 1994, 2002). *Arabidopsis* was shown to follow this general trend and there is compelling evidence that many mRNAs are stored in preparation for translation during tube growth (Twell 2002). Developmental transcriptomic studies also prove the existence of a large number of stored mRNAs in mature pollen. These studies also revealed storage of pre-formed protein synthesis machinery. For example, most protein synthesis genes are down-regulated in mature pollen, and *Arabidopsis* pollen tubes are strictly dependent on ongoing translation (Honys and Twell 2004).

Post-transcriptional regulatory mechanisms also operate through small RNA pathways (Matranga and Zamore 2007), and a hypothesis that pollen may be defective in this system has been formulated based on transcriptomic analysis. The apparent absence of mRNAs encoding smRNA pathway components in mature pollen indicated inactivation of the smRNA pathway in maturing pollen grains (Pina et al. 2005). The extent to which smRNA pathways function during male gametophyte development remains to be established, but miRNAs have been detected by in situ hybridisation in mature pollen of tobacco (Válóczi et al. 2006). Their operation is also apparent from the effective application of co-suppression and hairpin based RNAi constructs to down-regulate specific genes in developing and mature male gametophytes (Gupta et al. 2002; de Groot et al. 2004; Takeda et al. 2006).

12.10 Integrating Genetic and Transcriptomic Data

Transcriptomic studies have provided a genome-wide view of the complexity of gene expression and its dynamics during male gametophyte development. Male gametogenesis is accompanied by large-scale repression of gene expression associated with the termination of cell proliferation and the selective activation of genes involved in maturation and post-pollination events. Development is also associated with major early and late transcriptional programmes and the expression of about 600 putative transcription factors. The data also highlight an important role for mRNA and protein storage in mature pollen. Overrepresentation of genes involved in cell wall metabolism, cytoskeleton and signalling further highlight the functional specialization of mature pollen in preparation for rapid directional pollen tube growth.

Although a growing number of genes have been identified in *Arabidopsis* with a demonstrated requirement at different stages of pollen development, saturation screening by segregation distortion remains a daunting task. Similarly, morphological screens for male gametophytic mutations are far from reaching saturation, given that almost all developmental mutations are represented by single alleles.

Estimates of the number of genes that are essential for male gametophyte development including the progamic phase range from 330 in T-DNA and 575 in Ds insertional screens (Johnson et al. 2004; Lalanne et al. 2004a, b). However, from transcriptomic data about 14,000 genes are estimated to be expressed at one or more stages of male gametophyte development (Honys and Twell 2004). With the establishment of large FST (Flanking Sequence Tag) databases (Schoof et al. 2004), the total number of insertions that need to be screened to achieve saturation is more realistic and similar to the total number of annotated genes in *Arabidopsis*. Genetic redundancy is clearly a limitation in simple genetic screens but, to combat this deficiency, transcriptome data can now be used to carefully select reverse genetics targets based on co-expression. This informed genetic analysis is beginning to be exploited and several examples of functionally redundant proteins have been identified (Table 12.1). This will be made even more effective by using RNAi technology, exploiting an increasing repertoire of cell- and stage-specific promoters that are active in microspores, germ cells and at late stages in pollen development (Engel et al. 2005; Rotman et al. 2005; Honys et al. 2006a). The integration of these new molecular tools with established genetic approaches and transcriptomic analyses heralds an exciting era in which it will be possible to assemble complex functional models of male gametophytic development and function.

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Chapter 13
Pollen Germination and Tube Growth

D.-Q. Shi and W.-C. Yang

13.1 Introduction

In higher plants, the sexual organs, e.g. anther and pistil, are separated spatially either within a flower in hermaphrodite plants or in different flowers as in monoecious plants. Successful fertilization requires temporally and spatially coordinated development of male and female organs and gametophytes. Mechanisms have also been evolved to ensure pollen survival in harsh environments and to deliver the male gametes to the female gametophyte enclosed within the ovule, which in turn is embedded in the pistil. Furthermore, crosstalk between the male and female gametophytes ensures the success of fertilization that occurs at high efficiency and in a controlled manner.

The advent of multicellular gametophyte and siphonogamy in higher plants is instrumental in improving efficiency of sexual reproduction. In species with tricellular pollen grains, like Arabidopsis and rice, mature pollen grains are composed of two small sperm cells and a large vegetative cell. However, in tobacco, maize and other species with bicellular pollen grains, mature pollen grains contain a large vegetative cell and a small generative cell that will precede the last mitosis to produce two sperm cells in the pollen tube. The pollen tube, a tubular structure that originates from the vegetative cell, will deliver the two sperms to the female gametophyte, a phenomenon called siphonogamy (Fig. 13.1). In addition to the cells in pollen grains, specialized pollen walls have been developed to protect the male gametes and to meet the requirement for dispersal and interaction with the pistil. With respect to the female gametophyte, it is composed typically of seven cells, namely an egg cell, two synergids, a central cell and three antipodal cells that often degenerate before fertilization (Yang and Sundaresan 2000). The egg,

D.-Q. Shi and W.-C. Yang
The CAS Key Laboratory of Molecular and Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China
e-mail: wcyang@genetics.ac.cn

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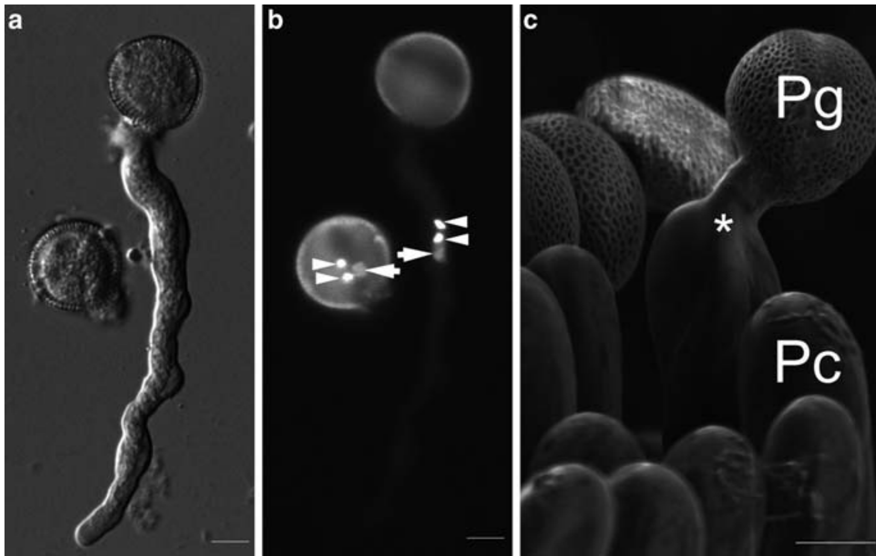

Fig. 13.1 Overview of pollen tubes in Arabidopsis. **a** Micrograph showing a germinating pollen (*left*) and pollen tube developing in vitro; **b** the same micrograph as in **a** showing sperm (*arrowheads*) and the vegetative nucleus as revealed by DAPI staining; **c** scanning electron micrograph showing a germinated pollen grain (Pg) and pollen tube (*) on a papillar cell (Pc). Scale bars in **a**, **b** or **c** are 10 μm

together with central cell and synergids, forms a functional female germ unit (FGU) to fulfil the task of pollen tube attraction, sperm release and transportation, and double fertilization. This chapter reviews our current understanding of pollen germination and tube growth, with a focus on Arabidopsis and other model species.

13.2 Mature Pollen Grains

As mentioned above, a tricellular pollen grain consists of two small sperm cells enclosed within a large vegetative cell, and together they form a functional assemblage, the male germ unit (MGU), which plays a potential role in the transport and delivery of sperm. The sperm and the vegetative cell can be easily distinguished by their size and nuclear morphology when pollen grains are released from the anther. The vegetative nucleus is highly lobed, while the sperm nucleus is elongated. Pollen grains undergo a maturation process prior to anther dehiscence to prepare themselves for survival, crosstalk with the female sexual organ, germination and tube growth. This process includes the elaboration of the pollen wall, dehydration and accumulation of storage materials.

13.2.1 Pollen Wall

The pollen wall is more complex than other cell walls in plants. It is composed of several layers, with a highly organized ultrastructure of each layer. The pollen wall is usually divided into three principle strata at maturity, namely the intine, exine and pollen coat, with the relative amount of each varying between species. The intine is the innermost wall made primarily of multilayered cellulose. It shares many of the chemical and structural features with the primary wall of a somatic cell. Immediately outside the intine is the reticulated exine that is composed of the chemically resistant biopolymer sporopollenin and interrupted by openings called apertures. The exine can be further divided into an outer sculptured layer, the sexine, and a simple inner layer, the nexine. Outside the reticulate exine layer resides the pollen coat that contains lipid, protein and other material deposited from the tapetum of the anther.

As the most specialized cell wall layer, the exine, provides the distinctive and characteristic features of pollen grains and spores. The exine pattern of the reticulate pollen wall is a unique feature of a species and can be divided into three major classes, namely baculate, granulate and spinulate. It has been important in palynology since the early 1970s because of its vast morphological diversity and composition of sporopollenin. Prior to the formation of the intine layer, exine formation is initiated from the tetrad stage during microsporogenesis. The glycocalyx-like fibrillar polysaccharide material, primexine, is deposited originally onto the microspore surface between the plasma membrane and the β-1,3-glucan (callose) wall. Subsequently, the precursors of sporopollenin, for example, the long-chain fatty acids, aromatic compounds, phenolics and carotenoid (Guilford et al. 1988) that are synthesized in tapetal cells, are polymerized and accumulated to form sporopollenin at raised sites of the plasma membrane, leading to the formation of the probacula within the primexine matrix. The prebacula elongates until it reaches the callose wall to form the bacula structure. At the end of the tetrad stage, as callose starts to dissolve, a structure called the tectum appears on top of the bacula, and simultaneously a foot layer is formed at the basal bacula (Paxson-Sowders et al. 1997, 2001). The primexine patterning requires coordination between the extracellular callose layer, the plasma membrane, and the underlying cytoskeleton, vesicles and endoplasmic reticulum. Callose deposition is fundamental to the exine patterning and formation. For example, mutation of a male-specific β-1,3-glucan synthase gene, *CalS5*, disrupts the exine organization (Nishikawa et al. 2005). It has been proposed that callose can increase the local concentration of primexine subunits by trapping these elements and preventing them from diffusing into other locules. In addition, callose can function as a physical support during primexine assembly or interacts directly with primexine nucleation sites.

Sporopollenin is a biopolymer that is resistant to non-oxidative physical, biological and chemical degradation processes (Blackmore 2007). Thus, as sporopollenin polymerizes, the exine becomes less elastic and increasingly resistant to acids, bases and enzymes. The spines or other characteristic structures that are

attributed to wide variety of taxa are formed at the surface of microspores. As exine patterning commencing from the tetrad stage continues, the aperture is set where the exine is much reduced or virtually absent between the plasma membrane and the adjacent callose wall. Apertures of exine are an important feature for taxonomic classification; they differ in number, distribution and architecture within families, species or even a single plant. Apertures are the sites for pollen tube exit; they also function in water uptake, transfer of recognition substances and accommodation of volume changes (Furness 2007). Despite morphological description, molecular mechanisms and factors controlling sporopollenin accumulation, aperture positioning and exine patterning remain to be clarified (Blackmore 2007).

The pollen coat refers to lipoidal substances covering mature pollen grains of entomophilous plants. The major substructure of the pollen coat is pollenkitt or tryphine, which refers to the extracellular lipidic substance that originates from plastids of tapetal cells and that covers the outer surface of the exine. Pollenkitt and tryphine may appear identical, although they differ in ontogenesis (Pacini 1997). The pollen coat is composed of lipids, proteins, pigments and aromatic compounds that fill the sculptured cavities of the pollen exine. A large proportion of the coat lipids are very long-chain lipids (C29 and C30) that account for about 70% of the coat lipids (Mayfield and Preuss 2000). In Arabidopsis, lipases and oleosin-like proteins amount to >90% of the detectable pollen coat proteins larger than 10 kD (Mayfield et al. 2001). Together, pollen coat substances account for 10–15% of total pollen mass (Piffanelli et al. 1997). Besides the protective function against environmental attack, the lipids and proteins of the pollen coat mediate the hydration of pollen grains on stigma cells and the recognition between pollen and the stigma. Proteins in the pollen coat may also act as ligands and receptors in this recognition system and/or components needed for efficient pollination. The cysteine-rich protein (SCR) residing in the pollen coat was discovered as the male recognition factor in self-incompatibility (SI) in Brassicaceae (Schopfer et al. 1999; Shiba et al. 2001). In Arabidopsis pollen, the major coat protein is an oleosin-like coat protein, GRP17-1. Knockout of *GRP17-1* causes delay in pollen hydration and reduced transmission, suggesting that GRP17-1 is essential for initiation of pollen hydration on the stigma and for pollen fitness. However, the molecular mechanism of GRP17-1 function in pollen hydration is not clear. Oleosin, a major component of the oil body in seeds of oil crops, plays a role in preventing oil body fusion during seed dehydration. It is not clear whether the oleosin-like proteins play a similar role for the pollen coat. They may either communicate directly with the stigma or mediate the biophysical properties of the pollen coat (Mayfield and Preuss 2000).

13.2.2 Pollen Maturation

Before anther dehiscence, pollen undergoes a maturation process, which prepares the grain for independent living and subsequent activities for sexual reproduction.

The maturation processes include pollen dehydration and accumulation of storage materials.

13.2.2.1 Pollen Dehydration

Mature pollen grains represent an arrested developmental stage in the life cycle of higher plants because of dehydration before dispersal. Dehydration is the loss of a certain amount of water from fresh grains. Pollens of most plant species (70%) undergo dehydration before anthesis. Pollen grains vary considerably in their degree of dehydration at dispersal, depending on their habitat. Dehydration causes physical changes, such as in size and shape, influencing speed of pollen transport, as well as harmomegathic changes in the appearance of the pollen wall. More importantly, pollen dehydration maintains pollen grains in a "dormant" or "quiescent" state, because the water content is approximately 15–35% that of active metabolism, and therefore increases the ability to withstand changes in environmental conditions after dispersal. Dehydration is thus highly related with pollen viability and composition of reserves within the grains. In *Cucurbita pepo* and several species of Gramineae, pollen grains are dehisced in a hydrated state and remain metabolically active. Thus, they are short-lived (Nepi and Pacini 1993). Water is tied up with membrane components during dehydration and reaches a threshold point at the end of dehydration. Consequently, the phospholipid material of the membrane system is converted from a liquid crystalline form to a gel state. In addition, the degree of dehydration is highly correlated with contents of carbohydrates and lipids in mature pollen grains.

13.2.2.2 Reserves in Mature Pollen Grains

The reserves in mature pollen grains are mainly carbohydrates, which are used as nutrient storage for energy resource or synthesis of pollen tube wall precursors. Proteins are usually absent in the reserves or their concentrations are lower than that in seeds. Pollen carbohydrate reserves at maturity may exist in different forms: (1) polysaccharides such as starch in amyloplasts or polysaccharides in cytoplasmic vesicles, (2) disaccharides such as sucrose and (3) monosaccharides such as glucose and fructose (Pacini 1996). Starch serves as an energy supply or is converted to other substances during pollen development and maturation (Kuang and Musgrave 1996). In most members of the Graminaceae, pollen grains contain a large amount of starch, with only a small quantity of soluble sugars, and are only slightly dehydrated at anthesis. The pollen viability decreases sharply after dispersal. In *Chamaerops humilis*, *Malus domestica*, *Mercurialis annua*, *Prunus avium*, *Ricinus communis*, *Trachycarpus excelsa* and *Typha latifolia*, pollen dehydrates before anthesis and is often of small or medium size. This type of pollen is characterized by long duration of pollen survival after dispersal. It contains high concentrations of soluble sugars and cytoplasmic polysaccharides with little or no starch. In *Lilium*,

Magnolia grandiflora, *Passiflora caerulea* and *Pinus halepensis*, pollen grains contain intermediate quantities of starch, cytoplasmic polysaccharides and soluble sugars, which are associated with intermediate longevity (Speranza et al. 1997).

In addition to the lipidic matrix of pollen walls and intracellular membrane systems, large amounts of oil bodies are also found in the cytoplasm of pollen grains in some species, such as *Brassica napus* (Charzynska et al. 1989), *Gossypium hirsutum* (Wetzel and Jensen 1992) and *Arabidopsis thaliana* (Kuang and Musgrave 1996). In most cases, lipid bodies are present in the cytoplasm of vegetative cells. However, lipid bodies have also been observed in generative cells of lily pollen or only in the generative cell cytoplasm in *Polystachia pubescens*. The distribution and appearance of lipid bodies have been recognized as cytological markers for the monitoring of pollen cell fate (Park and Twell 2001). Lipids are reserves in pollen in a manner that is analogous to the accumulation of storage oil bodies in many seeds. Both the membrane and storage lipids of pollen grains provide the substrates and energy reserves for pollen germination and subsequent elongation of the pollen tube. In Arabidopsis, considerable amounts of lipid droplets are present throughout the vegetative cytoplasm in mature pollen grains (Kuang and Musgrave 1996).

13.2.2.3 Transcriptome and Proteome of Pollen Grains

Pollen grains of most species are metabolically quiescent. However, they can respond rapidly upon pollination by interacting with stigma cells, and germinating pollen tubes undergo rapid polar growth. Undoubtedly, the readiness of pollen grains depends on the "pre-stored" substances, especially transcripts and proteins. Recent molecular analysis on mature pollen grains of representative species, including Arabidopsis, maize, lily, tobacco and *Plumbago zelanica*, indicated that mature pollen expresses a comparatively reduced and unique set of genes that are tightly associated with their main task of delivering sperm to the embryo sac (Honys and Twell 2003; Lee and Lee 2003; Becker et al. 2003; da Costa-Nunes and Grossniklaus 2004). Transcriptome profiles of Arabidopsis pollen show that, although the total number of expressed genes decline as male gametogenesis progresses, the expression of genes that function in the cytoskeleton, vesicle trafficking, cell wall biosynthesis and regulation, ion dynamics, signal transduction and other aspects related to pollen germination and tube growth is up-regulated significantly. For example, expression of gene clusters related to cell proliferation declines as pollen matures, while genes involved in pollen maturation or even post-pollination events increase. Consistently, the repression of constitutive genes and activation of male gametophyte-specific genes associated with specialization of mature pollen in preparation for pollen germination and pollen tube growth are observed in pollen transcriptomes. Furthermore, genes involved in small RNA biogenesis are down-regulated in Arabidopsis pollen. Fifteen genes of small RNA pathways are absent in mature pollen, although they are active in vegetative tissues. These findings indicate that small RNA pathways may be inactivated in mature

pollen of Arabidopsis. The observations also support the hypothesis that the male gametophyte reduces the complexity of its transcriptome, compared with the sporophyte, and maintains a limited amount of transcripts essential for pollen germination and the later delivery of sperm (Honys and Twell 2003; Becker et al. 2003; Pina et al. 2005).

Proteome profiles of rice pollen indicated that mature pollen grains pre-synthesize proteins needed for pollen function. These analyses showed that proteins involved in cell wall metabolism, protein synthesis and degradation, cytoskeleton dynamics and carbohydrate/energy metabolism are abundant in germinating rice pollen. In addition, multiple protein isoforms are present in mature pollen, suggesting that posttranslational modifications are common in mature pollen. Since transcripts of genes involved in transcription and protein synthesis are in low abundance in mature pollen grains, as shown by transcriptome profiles, it is apparent that a large amount of proteins are stored (Dai et al. 2006, 2007). These results suggest that the stored mRNAs and proteins are critical for subsequent pollen activity.

13.3 Pollen-Stigma Interaction

The pistil is a female sexual organ, an elongated structure located in a central position in a flower. A fully developed pistil is composed of three parts, namely the uppermost stigma, basal ovary that contains ovules and a style that connects the stigma and ovary. Pollen grains released from the anther are deposited on the stigma surface either directly in selfing plants or indirectly by wind or insects in cross-pollinating plants. After germination at the compatible stigma, the pollen tube subsequently penetrates through the stylar transmitting tissues of the ovary, exits the placenta and travels along the funicular surface. It finally enters an ovule and then the embryo sac, in which the pollen tube tip bursts in the synergid to release the two sperm cells for double fertilization. The nonmotile sperms are delivered directly to the embryo sac by the pollen tube, which allows flowering plants to carry out sexual reproduction on land without the need for water (Hiscock and Allen 2008).

13.3.1 The Stigma

The stigma surface is the place where pollen grains adhere, hydrate and germinate. In flowering plants, the interaction between pollen and stigma is the first event that initiates the process of fertilization. Successful pollen-stigma communication and coordination are essential to the subsequent processes of pollination. Stigmas are classified as wet or dry, according to the amount of matrices secreted by the specialized papillar cells of the stigma surface. For example, grasses such as rice,

and crucifers such as Brassica species and Arabidopsis possess dry stigmas with an epidermis of many large, hairy papillae cells interacting directly with pollen grains, to accept compatible pollen and reject pollen that is incompatible or from foreign species. The surface of papillar cells is covered with an interrupted layer of waxy cuticle overlaid with a distinct proteinaceous pellicle of poorly defined molecular composition (Ciampolini et al. 2001; Hiscock et al. 2002). However, wet stigmas often contain three distinct zones, namely an epidermis with papillae, a subepidermal secretory zone and a parenchymatous ground tissue. Legumes and members of the Orchidaceae and Solanaceae families are typical wet stigma-type plants. Most exudates are produced by the cells of the secretory zone, and less secretion is derived from the stigmatic papillae of wet stigmas at pistil maturity (Kandasamy and Kristen 1987). Moreover, pollen and stigma structures are largely coevoluted, since plants of trinucleate pollen species usually have dry stigmas, while the binucleate pollen species are concomitant with wet stigmas (Edlund et al. 2004).

Transcriptome profiling of rice stigma indicates that stigma-specific genes share conserved roles in plants. The majority of genes that are specifically or preferentially expressed in stigmas of Arabidopsis and rice belong to the cell wall-related and signal transduction groups, indicating that these two classes of genes play conserved roles in the stigma. Other highly expressed genes in the rice stigma are related with auxin signalling pathways, transport functions and stress responses (Li et al. 2007). In Arabidopsis, stigma-specific genes have functions related to the development of the stigma epidermis, in pollen recognition, or in the promotion of adhesion, hydration and germination of pollen grains (Tung et al. 2005).

13.3.2 Pollen Recognition

The pollen coat, as the first barrier for recognition, contains molecules involved in the initial interaction with the stigma (Mayfield et al. 2001). Proteins and lipids in the pollen coat act as ligands for receptors in the stigma, as seen in the self-incompatibility (SI) system exemplified in Brassica. The interaction between the pollen surface and the stigma differs from species to species, due to diversities in morphology and composition of the pollen coat and in the extracellular matrix (ECM) of the stigma. The interactions between pollen and dry stigmas are highly regulated and usually contain multiple steps of recognition, adhesion, rehydration and germination. The dry stigma of some species, such as crucifers, constitutes an important barrier for the rehydration of pollen grains. This barrier serves as an early discrimination for the male-female recognition system during pollination. To date, the best-characterized pollen-pistil interaction is the SI responses on dry stigmas in Brassica and on wet stigmas in Antirrhinum and petunia of the Scrophulariaceae. In the Brassica SI system, the male determinant, a pollen-secreted small cysteine-rich (SCR) protein, interacts with the female determinants, a cell wall S-locus glycoprotein (SLG) and plasma membrane receptor kinase (SRK) of the stigma

epidermal cells, to initiate an SI reaction (Schopfer et al. 1999; Nasrallah 2000). In contrast, in the wet stigmas, a wide range of proteins, and the lipidic exudate or carbohydrate-rich material that is produced by the cells of the secretory zone are secreted at pistil maturity. Also, a thin layer of water in crystal form surrounds the cells of the secretory zone underneath the exudates. Thus, the pollen grains on the stigma establish direct contact with the pistil through the exudates, while the hydration of pollen is facilitated by the carbohydrate- and lipid-rich secretions. Stigma secretion also plays a key role in pollen capture and adhesion. As a result, pollen is trapped rapidly and immersed within the secretion when contacting a wet stigma. It appears that the first interaction of pollen-stigma in wet stigmas is passive and indiscriminate (Swanson et al. 2004). Therefore, the inhibition of incompatible pollen may occur at later stages.

13.3.3 Pollen Adherence and Hydration

The pollen-stigma signalling machinery is critical for pollen recognition, and cell adhesion is likely required to stabilize pollen-stigma epidermal contacts. Adhesion of pollen grains onto the stigma surface is a key step, especially for plants with dry stigmas. Adhesion occurs prior to pollen hydration and germination, while the recognition may exist in one or through all the phases of adhesion, rehydration, pollen germination or even tube growth.

In Arabidopsis, pollen-stigma adhesion is rapid and highly selective. The binding force between pollen grains and stigma cells averages about 5.0×10^{-7} N. If, by analogy, this adhesive is spread onto an area of 0.1–0.5 m^2, it would be sufficiently strong to suspend a 100-kg object. Furthermore, the magnitude of pollen adhesion increases during pollination, and rapid remodelling of the stigma surface occurs simultaneously on adhesion. The stigma surface in contact with pollen grains protrudes into the space and the cell wall thickness changes. However, initial pollen-stigma adhesion is independent of the pollen coat. The adhesion molecules may exist in the exine, since the purified exine alone can bind to the stigma with high affinity (Zinkl et al. 1999).

After landing on the stigmatic surface of the pistil, the dehydrated pollen grains rehydrate, mobilize water from the papillar surface, activate their metabolism, and then germinate if the pistil is receptive. Adhesion and hydration are also complex and highly regulated in species with dry stigmas (Preuss et al. 1993; Dickinson 1995). When pollen grains fall on a compatible stigma, the exine coating changes dramatically in response to contact with the stigma surface and, subsequently, the coating flows out to form a “foot” to glue the pollen to the stigma (Elleman and Dickinson 1986). The “foot” differs from other parts of the pollen coat in some physical and chemical properties, particularly its insolubility in cyclohexane (Elleman et al. 1989). Furthermore, the hydration is dependent on pollen-pistil recognition and interaction. Compatible and incompatible pollen grains behave

differently if they are present on the same stigmatic papilla of Brassica. The compatible grains hydrate within 90 min, while the incompatible grains swell slightly by retaining their fusiform shape for up to 24 h. Only the sole hydration of the grain adjacent to the stigmatic papilla takes place whether the pollen grains are present on the stigma in chains or the "compound" pollinations are made with grains from other closely related species (Sarker et al. 1988). This implies that hydration occurs only between stigmatic papilla and pollen, but it cannot be passed amongst pollen grains. Post-pollination events occur rapidly in Arabidopsis. Apparent cytological changes take place within 5 min after pollen alights on the stigma. The hydration and regain of cytoplasm polarity in pollen grains and emergence of pollen tubes occur within 15 min after pollination. At about 20 min after pollination, pollen tubes can be visibly detected invading the expanded papillar cell wall and, after 40–50 min, pollen tubes have digested their way through both the separated outer and inner layers of the papillar cell wall and have reached the intercellular matrix separating the papillar cells from the subepidermal cells of the stigma (Kandasamy et al. 1994). However, in species with wet stigmas, the hydration of pollen grains is passive and autonomous. The stigma exudates are essential for pollen hydration, as they provide pollen grains with a medium containing not only water but also other materials for metabolism. Consistently, mutations eliminating exudate formation from stigmas result in female sterility in tobacco (Goldman et al. 1994; Wolters-Arts et al. 1998).

To address the signals controlling pollen adhesion and hydration, recent studies in Arabidopsis suggested that lipids might be the key signalling molecule. When triacylglycerides are added, pollen germination is restored on stigma-less pistils and the surface of other vegetative tissues, including leaves (Wolters-Arts et al. 1998). It has been reported that different lipids define the timing of pollen hydration, suggesting that lipids may mediate the permeability of the pollen grain surface to water (Wolters-Arts et al. 2002). Studies on *eceriferum* (*cer*) mutants of Arabidopsis confirmed the importance of pollen coat lipids for pollen hydration (Preuss et al. 1993; Fiebig et al. 2000). In Arabidopsis, *CER* genes form a large family involved in the biosynthesis of long-chain lipids that play a role in forming a hydrophobic waxy cuticle critical for resistance to water loss, pathogen attack or UV irradiation (Post-Beittenmiller 1996). All *cer* mutants (*cer1*, *cer2*, *cer3*, *cer6/pop1*, *cer8* and *cer10*) are unable to synthesize long-chain lipids and, consequently, result in male sterility due to lack of long-chain lipids in the pollen coat (Koornneef et al. 1989; Preuss et al. 1993; Hannoufa et al. 1996; Millar et al. 1999; Fiebig et al. 2000). Further studies show that the lack of lipid in the pollen coat interferes with pollen hydration but not adhesion. Nevertheless, the male fertility of *cer* mutants can be restored with high humidity, exogenous application of various triacylglycerides, mineral oil and a nonspecific alkane (Hernandez-Pinzon et al. 1999). Similarly, mutation in *FACELESS POLLEN-1* (*FTP1*), a gene encoding an aldehyde decarbonylase involved in wax biosynthesis, resulted in a phenotype similar to *cer* mutations (Ariizumi et al. 2003). These results suggest that lipid of the pollen coat is vital for pollen hydration.

Apart from pollen coat lipids, proteins in the pollen coat are also critical for pollen hydration. Knockout of the *GRP17* gene, which encodes an oleosin-like protein present in the pollen coat, causes the delay of pollen hydration and substantial reduction of pollen fitness. This implies that GRP17 plays a role in pollen hydration (Mayfield and Preuss 2000; Mayfield et al. 2001). Other proteins, such as aquaporins, also play an important role in regulating pollen hydration. Aquaporins are a class of proteins that specifically facilitate the passive movement of water and/or small neutral solutes (e.g. urea, boric acid and silicic acid) or gases (e.g. ammonia and carbon dioxide) through membranes (Maurel et al. 2008). It has been reported that an aquaporin-like plasma membrane protein encoded by *MIP-MOP* (major intrinsic protein associated with the *MOD* locus) is abundant in papillar cells of the stigma, and may act as a major regulator of hydration by controlling water flow from the stigma to the pollen grains (Dixit et al. 2001).

During pollen hydration, the papillar cell is thought to actively transport water, calcium, boron and other components essential for pollen germination into the pollen grains. As pollen grains hydrate, extracellular Ca^{2+} flows into the grains. This influx appears to trigger the rapid activation of the stored RNA, protein and bioactive small molecules that allow rapid germination and tube growth (Mascarenhas 1993).

13.4 Pollen Germination and Tube Growth

Hydration causes changes in the water content and volume of the pollen grain, which may act as the initial signal to trigger pollen germination. As hydration occurs, Ca^{2+} influx takes place and initiates the reorganization of the cytoskeleton and also polarizes the cytoplasm of the vegetative cell in the pollen grain. Subsequently, cytoplasmic polarity of the vegetative cell is established, as manifested by local accumulation of vesicles and a cytoplasmic gradient of Ca^{2+} beneath the pore near the site of adhesion (Kandasamy et al. 1994; Franklin-Tong 1999a, b). Consequently, the vegetative cell germinates to produce a pollen tube. The aperture is often the site where the pollen tube emerges. However, for inaperturated pollen grains, the pollen tube may exit anywhere.

The pollen tube wall is an extension of the intine, and tube elongation is the rapid tip growth of a single cell growing directionally from one end. Establishment and maintenance of the polarity of the tube are important to attain rapid polar tip growth. Consistently, gene products involved in cell rescue, transcription, subcellular localization, metabolism, proteins with binding function, and cellular transport are overrepresented during pollen germination, except for the "pre-stored" transcripts and proteins in pollen. Similarly, these genes are also up-regulated during pollen tube growth (Wang et al. 2008). Also, it has been well documented that calcium, the cytoskeleton, small GTPases and other factors regulating polar growth are critical for pollen germination and tube growth.

13.4.1 Calcium Signalling in Pollen Germination and Tube Growth

As a universal signalling molecule, Ca^{2+} is essential for pollen tube growth. The role of calcium has been demonstrated in several studies. Firstly, accumulation of cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyt}$) at the germinal aperture, where the pollen tube emerges, occurs soon after hydration and calcium influx takes place at the pollen tube tip (Franklin-Tong et al. 2002; Lazzaro et al. 2005; Bushart and Roux 2007). Secondly, if no $[Ca^{2+}]_{cyt}$ gradient is established in the pollen grains, there is no protrusion following hydration, and germination is inhibited. Inhibition of calcium channels can also block pollen germination in vitro (Franklin-Tong et al. 2002; Wang et al. 2004; Bushart and Roux 2007). Thirdly, pollen tube growth is arrested when Ca^{2+} uptake is inhibited (Jaffe et al. 1975; Bednarska 1989; Franklin-Tong 1999a). The germination and reorientation of pollen tubes have been shown to parallel calcium concentration. Disruption or modification of the Ca^{2+} gradient at the tube apex interrupts tube growth (Miller et al. 1992) or induces bending of the growth axis towards the zone of higher $[Ca^{2+}]_{cyt}$ (Malhó and Trewavas 1996). These findings clearly show that calcium is essential for pollen germination and tube growth in vitro. Interestingly, concomitant with a $[Ca^{2+}]_{cyt}$ increase in the pollen grain, a $[Ca^{2+}]_{cyt}$ gradient of the papillar cell can also be induced by pollination (Iwano et al. 2004), suggesting that interaction between pollen grain and papillar cells during pollination is mediated through calcium-dependent signalling.

How does Ca^{2+} exert its control over tip growth? The regulation of $[Ca^{2+}]_{cyt}$ dynamics is a combined effect of ion pumps, antiporters and uniporters. The dynamics of the Ca^{2+} signal is controlled by influx (through channels) and efflux (through pumps and antiporters; Schiøtt et al. 2004). Influx of Ca^{2+} is limited to a small region at the tube apex (Pierson et al. 1996; Holdaway-Clarke et al. 1997), and this tip-localized entry appears largely responsible for the formation of the Ca^{2+} gradient. Also, Ca^{2+} signalling is changed when efflux is blocked by the disruption of the Ca^{2+} pump or by antiporters (Schiøtt et al. 2004). Besides the formation of a calcium gradient along the growing tip, $[Ca^{2+}]_{cyt}$ oscillation may likely play a role, as fluctuations in $[Ca^{2+}]_{cyt}$ have been observed to correlate with pulses in tube growth (Holdaway-Clarke and Hepler 2003; Malhó et al. 2006). It has also been reported that $[Ca^{2+}]_{cyt}$ ranges from 2 to 10 μM at the apex, decreasing sharply to 20 to 200 nM within 20 μm down the apex (Obermeyer and Weisenseel 1991; Rathore et al. 1991; Miller et al. 1992; Malhó et al. 1994; Pierson et al. 1994; Franklin-Tong et al. 1997). Therefore, it has been speculated that both the concentration gradient and oscillation of $[Ca^{2+}]_{cyt}$ are important signals, which may function via the Ca^{2+} sensor calmodulin (CaM) and its downstream effectors, such as CaM-binding proteins in the pollen tube. In Arabidopsis, mutations in *NO POLLEN GERMINATION1* (*NPG1*) and *NPG1-related* genes, coding for CaM-binding proteins, abolish pollen germination (Golovkin and Reddy 2003), confirming a role of Ca^{2+} signalling in pollen germination.

How the Ca^{2+} signalling leads to polar tube growth remains to be elucidated, although a large number of proteins involved in Ca^{2+} signalling have been detected in the pollen transcriptome (Honys and Twell 2004; Pina et al. 2005; Becker and Feijó 2007). Recent studies showed that calcium-dependent protein kinases (CDPKs), such as the calmodulin-like domain containing protein kinases, are likely the candidate modulators of Ca^{2+} signalling (Yoon et al. 2006). Pollen-specific CDPKs have been identified from maize and petunia, and they may serve as Ca^{2+} regulators in pollen tube growth (Estruch et al. 1994; Moutinho et al. 1998; Yoon et al. 2006). Inhibition of CDPK activity with kinase inhibitors or calmodulin (CaM) antagonists has been shown to impair pollen germination and tube growth. CaM has been considered as a primary sensor of the Ca^{2+} signal regulating the activity of many proteins, such as enzymes, cytoskeletal and structural proteins, via its Ca^{2+}-binding capacity. Consistent with the polar distribution of Ca^{2+}, CaM accumulates at the germinal aperture, and a tip-base CaM gradient exists in elongating pollen tubes (Hauber et al. 1984; Tirlapur et al. 1994). In tobacco, CaM accumulates in the cytoplasm close to the three germinal apertures of the pollen grains, particularly near the aperture from which the pollen tube emerges. Furthermore, directional migration of CaM has also been observed during pollen hydration and germination, suggesting that the dynamic patterns of CaM distribution may play a crucial role in the establishment of polarity during pollen germination (Tao et al. 2004). In the growing pollen tube, the binding capacity of CaM appears more intense in the subapical region, indicating a higher concentration of CaM targeting molecules, such as cytoskeletal elements, in this region. Microfilaments resembling the V-shaped collar reported for CaM binding are present at the growing tip (Miller et al. 1996; Moutinho et al. 1998), suggesting a close link between the actin cytoskeleton and calcium signalling during pollen germination and tube growth. In addition, CaM may crosstalk with the cAMP signalling pathway, as CaM binds cyclic nucleotide-gated channels (CNGC), the mutation of which affects polarized tip growth of pollen tubes in Arabidopsis (Frietsch et al. 2007). Expression of *CNGC18* in *Escherchia coli* resulted in a time- and concentration-dependent Ca^{2+} accumulation, indicating that CNGC may transduce the cyclic nucleotide signal into a Ca^{2+} influx, thereby regulating polar tip growth. In yeast and animals, it has been well established that CaM controls the actin cytoskeleton via regulation of phosophatidylinositol 4,5-bis-phosphate (PIP_3) synthesis (Desrivieres et al. 2002), but a similar link in plants remains to be elucidated.

13.4.2 The Cytoskeleton

Cytoskeleton dynamics regulate many important cellular processes, such as organelle movement, chromosome segregation, flagellar movement and pollen tube elongation. A hallmark pollen tube feature is its cytoskeleton, which comprises actin filaments and microtubules (MTs). Microfilaments (MFs) and MTs have long been recognized as important structures for pollen germination and tube growth.

However, the ability to dissect the distribution and dynamics of the cytoskeleton in pollen tubes growth either in vivo or in vitro became feasible only after the availability of immunocytochemical and in vivo GFP-labelling methods in plant research. Based on cytoskeleton organization, the pollen tube can be divided into three main regions, namely the shank, subapical and apical regions. Observations of chemically fixed and living pollen tubes reveal the elaborate cytoskeleton constituting an extensive matrix of actin and microtubule bundles along the shank of the tube, reaching the subapical region, but hardly at the apical dome (Kost et al. 1998; Fu et al. 2001; Chen et al. 2002; Lovy-Wheeler et al. 2005; Cheung and Wu 2008). As a distinct structural and functional domain in elongating pollen tubes, the subapical region is characterized as a network of interdigitating actin cables, and a dramatic dynamics of organelles is associated with this actin mesh in this region (Chen et al. 2002; Cheung and Wu 2004). Although mitochondria and Golgi bodies have been observed throughout the pollen tube, the highest concentration is found in the subapical region. Tubular endoplasmic reticulum is concentrated mostly in the apical region, whereas rough endoplasmic reticulum is abundant, starting behind the inverted cone region and throughout the rest of the cytoplasm (Cheung and Wu 2007). The apical cytoplasm of the tube is the so-called clear zone, which lacks light-reflecting organelles (Steer and Steer 1989; Pierson et al. 1990). This zone is packed with secretory vesicles and recycled endocytosed membrane (Camacho and Malhó 2003; Parton et al. 2003). Many of the vesicles in the clear zone fuse with the apical membrane, thereby adding new membrane, membrane proteins, cell wall materials, secretory proteins and other signal molecules for the rapid growth of the tube (Luu et al. 2000; de Graaf et al. 2005).

13.4.2.1 Actin Cytoskeleton

As adaptive to the tip-growing character of the pollen tube, the cytoskeleton of the polar cell develops special features for rapid polar growth and the delivery of sperm cells in the tube, including specific cytoskeleton-binding proteins and polar configuration. The polarization of actin filaments and microtubules are initiated as hydration and germination of pollen grains occur. Subsequently, MFs and MTs are organized as longer bundles and enter the emerging tube (Tiwari and Polito 1990). In the elongating pollen tubes, actin filaments and MTs are structured in bundles that extend along the longitudinal axis reaching the subapical region. These occur both in the cortical and central cytoplasm, as well as in the apical dome of the pollen tube. A dynamic form of tip-localized short actin bundles (SABs) and a striking cortical fringe of F-actin have been observed in tobacco and lily pollen tubes with the aid of GFP-tagged actin-binding domain of mouse talin (Fu et al. 2001; Lovy-Wheeler et al. 2005). In lily pollen tubes, the actin fringe starts a few microns back from the tip and extends basally for an additional 5 to 10 μm. It seems that this fringe is fragile and difficult to preserve. However, the key structure is likely to perform rapid remodelling and polymerization and, subsequently, plays a central role in establishing cell/cytoplasmic polarity and in controlling rapid,

oscillatory growth in lily pollen tubes (Cárdenas et al. 2008). Consistently, the dynamics of actin both in the apical and subapical region correlates closely with the tip growth of pollen tubes (Cheung and Wu 2004). The prevention of actin polymerization with low concentrations of latrunculin B or enhancing actin polymerization profoundly affects pollen tube growth (Cárdenas et al. 2005; T. Chen et al. 2007). Furthermore, the strictly regulated actin dynamics and the level of nascent actin filament production seem critical to maintain tip growth (Vidali et al. 2001; Chen et al. 2002; Cheung and Wu 2004).

In tip-growing cells, such as pollen tubes and root hairs, cytoplasmic streaming, a process dependent on the actin cytoskeleton, is critical for rapid growth. For example, cultured tobacco pollen tubes possess a diameter of ~10 μm, but the tubes can elongate at an average rate of 5 μm min^{-1} and grow to a length of up to 15 mm, which is 1,500 times their diameter (Parton et al. 2003; Kost 2008). The rapid tip growth depends on a continuous supply of secretory vesicles containing cell wall material, which fuse with the plasma membrane specifically at the apex, manifested by a constant flow of organelles towards the apex and vice versa (Derksen et al. 1995; Campanoni and Blatt 2007). A bidirectional, "reverse fountain" pattern of cytoplasmic streaming is a hallmark of growing pollen tubes in angiosperms. During the streaming, the forward lanes flow along the edge of the cell but, in the apex, they undergo a reversal in direction, and stream basally through the pollen tube interior. The cytoplasmic streaming allows the transport of cargo particles or secretory vesicles, such as Golgi vesicles, carrying pectin and many other cell wall components to the rapidly growing cell (Cai et al. 1997; Franklin-Tong 1999a). The transport is thought to be driven by myosin on the vesicle surface that moves along the actin microfilaments (Miller et al. 1995; Yokota et al. 1995). Actin MFs in the shank are organized into bundles with uniform polarity and serve as tracks for the tip-ward streaming. However, in the subapical region, F-actins are less organized into fine filament bundles and form a collar-like zone that contributes to the reversed streaming and, consequently, gives rise to a reverse-fountain pattern (Hepler et al. 2001; Li et al. 2001; Ren and Xiang 2007). The actin MFs also play a critical role in the reversal of streaming in the pollen tube apex (Cárdenas et al. 2005).

The dynamics of the actin cytoskeleton is tightly regulated in growing pollen tubes, especially in the apical and subapical regions. The formation of F-actin arrays depends on the biochemical interactions of actin monomers and actin-binding proteins (ABPs). The elaborate architecture of the actin cytoskeleton is a result of the fine spatial and temporal incorporation of ABPs. Therefore, ABPs regulate the depolymerization and polymerization of actin filaments, equilibrate the globular actin (G-actin) and F-actin pools, and perceive or transduce various signals that affect the cytoskeleton, such as Ca^{2+}, pH, phosphoinositides and phosphorylation. ABPs are divided into several groups according to their function and structure. These include profilin, actin-depolymerizing factor (ADF) or cofilin, villin, gelsolin or fragmin, actin nucleators and heterodimeric capping proteins (Hussey et al. 2006; Ren and Xiang 2007; Xiang et al. 2007; Cheung and Wu 2008; Qualmann and Kessels 2008). Profilin is distributed throughout the pollen tube cytoplasm.

It binds monomeric (G-) actin, modulates actin nucleation and enhances actin polymerization. Subject to the high Ca^{2+} concentrations in the apical region, more actin-profilin complexes may form at the tip (Kovar et al. 2000; Vidali et al. 2001; Snowman et al. 2002). ADF/cofilin binds to both the monomeric and F-actin to enhance actin depolymerization by increasing the off-rate of actin monomer from the pointed ends, and inducing filament severing (Chen 2002). Its binding activity is regulated by phosphorylation (Allwood et al. 2001), pH and phosphatidylinositol 4,5-bisphosphate (PIP2) or phosphatidylinositol 4-monophosphate (PIP; Gungabissoon et al. 1998). ADF serves as actin filaments at high pH (8.0) and binds to F-actin at a lower pH of 6.0 (Gungabissoon et al. 2001; Yeoh et al. 2002). Villin has been shown to assemble actin filaments into bundles in a unipolar manner. It is also one of the Ca^{2+}-regulated ABPs, as it is negatively regulated by a Ca^{2+}-calmodulin complex (Yokota and Shimmen 2000). In the tube apex, as the Ca^{2+} concentrations are greater than in other regions, the villin activity is inhibited and actin bundles are not detected. This finding is consistent with the results of previous studies showing that only a mesh of short actin filaments, but not actin bundles, is found in the pollen tube apex (Miller et al. 1996; Kost et al. 1998; Yokota and Shimmen 2000). Gelsolin, fragmin and severin are members of the gelsolin family that perform severing, capping and nucleating activity in a Ca^{2+}-dependent fashion (Staiger and Hussey 2004). Gelsolin-like proteins have been identified in maize, lily and poppy pollen. In poppy pollen, PrABP80, a Ca^{2+}-regulated gelsolin-like ABP, can nucleate actin polymerization from monomers and block the assembly of the profilin-actin complex onto actin filament ends, thereby enhancing profilin-mediated actin depolymerization (Huang et al. 2004). It has also been observed that the Arabidopsis heterodimeric capping protein (AtCP) prevents the addition of profilin actin to barbed ends, being sensitive to phosphatidylinositol 4,5-bisphosphate. In the presence of phosphatidic acid (PA), AtCP is unable to block the barbed or rapidly growing and shrinking end of actin filaments. Inhibition of CP activity in cells by elevated PA results in the stimulation of actin polymerization from a large pool of profilin-actin, suggesting that capping proteins may imply a link to lipid-regulated polar cell growth (Huang et al. 2006). The roles of ABPs in pollen tube growth require further investigation.

13.4.2.2 Microtubule Cytoskeleton

While the role of the MT cytoskeleton in pollen tube growth is controversial, it has been reported that MT-depolymerizing chemicals (such as colchicine) do not inhibit organelle movement in the pollen tube (Heslop-Harrison et al. 1988). However, MT perturbation has been shown to cause the loss of cytoplasmic organization (Joos et al. 1994), and the pulsed growth rate of tobacco pollen tubes is modified when MT is depolymerized (Geitmann et al. 1995). The ability to maintain the growth direction of the pollen tube can be affected by MT degradation, while the integrity of the MT cytoskeleton depends on the presence of actin filaments. However, the actin filaments appear to be independent of the MT

configuration (Gossot and Geitmann 2007). Both the MT and the actin cytoskeleton are involved in organelle movement. The mitochondria and the Golgi apparatus have been shown to move slower along MTs than is the case for actin MFs (Romagnoli et al. 2007), indicating that MTs play a role in the positioning of organelles. It has been proposed that MTs mediate the migration of the male germ unit and the trafficking of vesicles and organelles either independently (Astrom et al. 1995; Romagnoli et al. 2003) or through interaction with actin filaments (Cai et al. 1997, 2000; Gossot and Geitmann 2007). Recently, the role of MTs in polar growth was demonstrated in fission yeast, *Schizosaccharomyces pombe*. MTs were shown to mediate the recruitment or accumulation of polar factors to the growing tip, leading to a polarity alteration of cell growth in the physically bent fission yeast cells (Minc et al. 2009). It remains to be investigated whether MTs have a similar role in pollen tube growth.

13.4.3 Crosstalk Between Calcium Signalling and Cytoskeleton in the Pollen Tube

The polar growth of the pollen tube requires the continuous delivery of new materials carried by secretory vesicles to the apex of the tube, while pollen tubes exhibit oscillatory growth. In Arabidopsis pollen tubes, the pollen tube growth rate increases following the actin-dependent and oscillatory motion of the endoplasmic reticulum (ER). It has been well documented that actin dynamics underlies the mechanism that initiates the surge in growth (Fu et al. 2001; Lovy-Wheeler et al. 2007). As discussed above, the configuration of actin filaments is controlled by a variety of ABPs, most of which are regulated by the Ca^{2+} signal. Ca^{2+} is a central factor controlling the transition from G-actin (or short actin mesh) in the tube apex to the F-actin cables in the shank. Furthermore, Ca^{2+} controls the fusion of vesicles and dynamics of actin filaments through its diverse activities and interactions with ABPs involved in actin bundling, severing, capping and nucleating. It has been reported that myosin-mediated organelle movement along actin bundles is inhibited by high Ca^{2+} concentrations at the apex, facilitated in the base region of the pollen tube by lower Ca^{2+} concentrations. Besides the regulation of ABPs by Ca^{2+} concentrations, Ca^{2+} channels, together with channel-mediated Ca^{2+} influxes across the plasma membrane of pollen and pollen tubes, are closely associated with actin MFs. In Arabidopsis, Ca^{2+} channel blockers, La^{3+} and Gd^{3+}, and F-actin depolymerization reagents significantly inhibit pollen germination and tube growth (Wang et al. 2004). The inhibition of pollen germination and tube growth by cytochalasins is enhanced by an increase in external Ca^{2+}. In contrast, Lat-B inhibition on pollen tube growth is coupled closely with a decline in the apical Ca^{2+} gradient (Cárdenas et al. 2008). Similarly, pollen tubes exhibiting non-oscillating growth display a reduced and non-oscillating Ca^{2+} gradient. This reflects either the different modes of action amongst the inhibitors or the pleiotropic effects of Lat-B. Lat-B can cause

a number of reversible phenotypes, including elimination of the acidic domain at the extreme tube apex, a shift of the alkaline band towards the tip, degradation of the cortical fringe, disorganization of the microfilaments in the shank, and obliteration of the clear zone with the organelles invading into and through the extreme apex of the tube. These results suggest that there may be crosstalk between the actin cytoskeleton and Ca^{2+} signalling in controlling pollen tube growth (Cárdenas et al. 2008).

13.4.4 Small GTPases and Pollen Tube Growth

Small GTP-binding proteins are molecular switches that can cycle intrinsically between an "active" GTP-bound form and an "inactive" GDP-bound form, depending on their substrate. This reversible reaction is modulated by proteins that affect the rate of either GTP hydrolysis or GDP release, thereby operating the switch. These small G proteins represent a conserved signalling pathway in eukaryotes, and control a variety of cellular processes, including polar cell growth. Guanine nucleotide exchange factors (GEFs) activate membrane-associated GTPases by exchanging GDP with GTP, while GTPase-activating proteins (GAPs) promote GTP hydrolysis to inactivate GTPases. Guanine nucleotide-dissociation inhibitors (GDIs) have been shown to inhibit the activation of GTPases by suppressing nucleotide exchange and sequestering GTPases into the cytosol. The small GTPases in the Ras-like small GTPase superfamily are classified into five distinct families, according to their structure and function. These are Ras, Rab, Rho, Arf and Ran. Ras GTPases regulate cell proliferation in yeast and mammalian systems, but they are evolutionally lost from some lineages, including plants. Rho GTPases are involved in actin dynamics and signal transduction pathways involving MAP kinases (Vernoud et al. 2003). Plants have a novel and unique family of Rho-like GTPases (ROPs), represented by a large number of proteins (Zheng and Yang 2000; Valster et al. 2000). Since plant ROPs are similar to animal Rac proteins in primary sequence, and interact with proteins bearing the "CRIB" (Cdc42/Rac-interactive binding) domain, they are also referred to as RAC proteins, Rac-like GTPases or Rac-Rop GTPases (Vernoud et al. 2003; Kost 2008). ROPs regulate actin dynamics and modulate H_2O_2 production in polarly growing cells and defence responses (Yang 2002; Morel et al. 2004). The Rab and Arf (ADP-ribosylation factors) GTPase families participate in endosomal vesicle trafficking, and Ras-related nuclear protein (Ran) GTPases function in nucleo-cytoplasmic transport of proteins and RNAs (Vernoud et al. 2003). Small GTPases are often lipid-modified and membrane-localized, with the exception of Ran GTPases (Molendijk et al. 2004). Hence, among the proteins associated with GTPases, there are docking/scaffolding proteins that target GTPases to specific membranes or membrane domains, or as GDI-displacement factors (GDFs) to reinforce the re-association of GTPases with the membrane (Fauré and Dagher 2001; DerMardirossian and Bokoch 2005).

Here we focus on the role of GTPases, especially Rab GTPases and ROPs, in pollen tube growth in plants.

13.4.4.1 Rab GTPases

In pollen tubes, small GTPases have been implicated in at least two vital functions, namely regulation of membrane vesicle trafficking and regulation of actin microfilaments (Krichevsky et al. 2007). Rab GTPases function crucially in membrane fusion processes in the pollen tube (Molendijk et al. 2004; Cole and Fowler 2006). As discussed above, rapid and polarized pollen tube growth demands an active secretory system, which can provide sufficient material for the rapid elongation of the tube. Hence, exocytosis responsible for the transport of secretory vesicles, small signal molecules or enzymes is essential for pollen tube growth. Also, endocytosis that may contribute to recycling and retrieval of the excess cellular material also occurs at the growing tip of pollen tubes (Carroll et al. 1998; Krichevsky et al. 2007). There is increasing evidence showing that Rab GTPases play a pivotal role in intracellular membrane trafficking, including endomembrane trafficking, exo/endocytosis and membrane recycling (Surpin and Raikhel 2004; Molendijk et al. 2004). It is likely that Rab GTPases interact with SNARE proteins present in organelle membranes and vesicles, in guiding these to their correct destiny and providing specificity for membrane fusion (Stenmark and Olkkonen 2001; Zerial and McBride 2001; Pfeffer and Aivazian 2004). Individual members of Rab GTPases are localized to different cellular membrane compartments. These members organize intracellular membrane trafficking at three consecutive stages, namely membrane budding and vesicle formation, tethering vesicles to target areas and promoting fusion of vesicles with target membranes (Tamm et al. 2003; Seabra and Wasmeier 2004).

In gaining insight into the Rab GTPase function in pollen tube growth, genetic approaches are greatly hampered by the overlapping functions of members of the Rab family. Instead, a dominant-negative approach is employed. This approach involves changes in Rab GTPases induced by introducing amino acid mutations either stabilizing the GTP-bound activated state and up-regulating the regulatory activity of Rab GTPases, referred to as constitutively active (CA), or locking the GDP-bound inactivated form as dominant-negative (DN). Studies based on CA or DN mutations of Rab GTPases in Arabidopsis and tobacco have revealed the functions and regulatory pathways of Rab proteins, which are highly conserved among yeast, animals and plants (Batoko et al. 2000; Grebe et al. 2003; Preuss et al. 2004; Ueda et al. 2004; de Graaf et al. 2005). A pollen-predominant Rab2 from tobacco, NtRab2, has been shown to function in vesicle trafficking between the ER and Golgi bodies, transporting cell membrane and secretory proteins. DN mutations in NtRab2 inhibit the transport of pollen proteins entering the secretory pathway, thereby suppressing pollen tube elongation. This implies that protein secretion in pollen tubes relies on Rab2 GTPase that may function specifically in ER-to-Golgi trafficking in highly secretory cell types (Cheung et al. 2002). Similarly, NtRab11b,

a member of the Rab11 subfamily of tobacco, is localized predominantly to the transport vesicle-occupied apex of elongating pollen tubes, suggesting that it may be involved in the apical accumulation of transport vesicles (Derksen et al. 1995; Hepler et al. 2001). DN or CA expression of NtRab11b inhibits pollen tube growth, disrupts growth directionality and causes male fertility. In addition, F-actin organization in the apical and subapical regions of pollen tubes is also affected when NtRab11b activity is misregulated.

Unlike yeast and animals, only a few Rab GTPase effectors have been identified in plants. A recent study has shown that PI-4 Kβ1, an effector of RABA4b in Arabidopsis, plays a role in the polarized expansion of root hair cells (Preuss et al. 2006). However, the role of PI-4 Kβ1 in pollen tube growth remains to be elucidated.

13.4.4.2 Rho-Like GTPases

In addition to Rabs, the Rho GTPase family has also been reported to be involved in pollen tube growth. Rho GTPases are Ras-related small guanine nucleotide-binding proteins that bind GTP and GDP with high affinity, but hydrolyze GTP inefficiently (Yalovsky et al. 2008). Similarly to the GTPases of other families, the activity and physiological status of Rhos are also modulated by the accessory binding proteins. As key nodes of signalling networks and central players of multiple pathways, Rho GTPases receive input signals from upstream regulators and then transduce to downstream effectors, thereby orchestrating a wide range of intracellular signalling networks. Like Rabs, Rho GTPases are also involved in regulating a variety of cellular processes, such as cell polarity, endocytosis and vesicle trafficking (Berken 2006; Nibau et al. 2006; Yang and Fu 2007). The most fundamental effects of Rho GTPases are on the actin cytoskeleton and dynamics. Rho signalling is often restricted to a specific domain of the plasma membrane, where vesicle exocytosis takes place (Fu et al. 2002; Gu et al. 2006; Hwang et al. 2008; Lee et al. 2008).

Rho family proteins in animals are functionally divided into five subfamilies, namely the Rho-like, Rac-like, Cdc42-like, Rnd and RhoBTB subfamilies, which have emerged as key regulators of the actin cytoskeleton and its dynamics (Burridge and Wennerberg 2004). However, the ROP family is a single, unique Rho-like GTPase from plants. The ROP family is known to regulate tip growth and polar cell expansion in plant cells. In Arabidopsis, there are 11 different ROP genes, seven of which are abundant or preferentially expressed in mature pollen (Vernoud et al. 2003; Honys and Twell 2004; Pina et al. 2005). More ROPs and their interacting proteins have been identified from results of studies on gain- or loss-of-function mutants (Gu et al. 2004; Hwang and Yang 2006). In particular, the pollen-specific AtROP1, which is localized to the plasma membrane in the apical region of the tube, has been shown to be a central switch and essential regulator of pollen tube tip growth. Blocking of ROP signalling by *DN-Atrop1* mutation inhibits pollen tube elongation, whereas *CA-Atrop1* mutation induced isotropic growth (Kost et al. 1999; Li et al. 1999). The dynamics of short actin

bundles (SABs) in the apex of tobacco pollen tubes is also regulated by AtROP1. Over-expression of *AtROP1* causes depolarized growth of the pollen tube, which can be rescued by over-expression of *GDI*, *RopGAP1*, or LatB treatment. Recently, it was shown that REN1 (ROP ENHANCER 1), a Rho GAP that localized to the apical cap and exocytic vesicles in the pollen tube tip, controls a negative-feedback-based global inhibition of ROP1 and regulates the dynamics of ROP1 through the restriction of active ROP1 to the apical cap (Hwang et al. 2008). These results provide direct evidence that Rho GTPase and actin organization are closely linked in cell polarity and tip growth in plants (Fu et al. 2001). Similarly, over-expression of a tobacco ROP/Rac protein, NtRac1, induces the actin cables to extend to the apical region, and causes tremendous disorganization of actin cables in the expanded tips (Chen et al. 2002). These findings suggest a central role of ROP in pollen tube growth.

PtdIns 4,5-P_2 is a downstream effector of ROP and a ligand for a large number of regulatory proteins, including actin-binding proteins and proteins with pleckstrin homology (PH-) or C2 domains. In tobacco pollen tubes, the ROP/RAC GTPase interacts physically with phosphatidylinositol monophosphate kinase (PtdIns P-K) and colocalizes with its product, PtdIns 4,5-P_2, in the apical membrane (Kost et al. 1999). PtdIns 4,5-P_2 might function both downstream and upstream of RAC/ROP activation. As an effector of Rac-Rop GTPases, PtdIns 4,5-P_2 promotes the fusion of secretory vesicles with the plasma membrane; on the other hand, PtdIns 4,5-P_2 might function as a positive regulator to stimulate the actin cytoskeleton and membrane trafficking by regulating the activity of ABPs and calcium homeostasis during tip growth (Janmey 1994; Kost et al. 1999; Kost 2008). In addition, PtdIns 4,5-P_2 is regulated by a pollen tube phosphoinositide-specific phospholipase C (PLC), which maintains the polar distribution of PtdIns 4,5-P_2 and Rho signalling. Studies on petunia PLC1 and tobacco NtPLC3 indicate a major function of PLC activity in the regulation of PtdIns 4,5-P_2 in the apical plasma membrane of elongating pollen tubes. It is likely that PLC activity maintains the spatially restricted RAC/ROP signalling and polarized cell expansion by preventing the lateral spreading of PtdIns 4,5-P_2 (Dowd et al. 2006; Kost 2008). Diacylglycerol (DAG) and the Ca^{2+}-mobilizing messenger $InsP_3$ are the two other cellular regulators released by PLC-mediated hydrolysis of PtdIns 4,5-P_2. While $InsP_3$ is involved in Ca^{2+} release from internal stores in plant cells, DAG is detected specifically in the plasma membrane at the tip of elongating pollen tubes (Helling et al. 2006). Furthermore, accumulation of DAG at the apex of pollen tubes is prevented in the presence of PLC inhibitors, suggesting that DAG accumulation may play an important role in RAC/ROP-dependent signalling, and that it may be linked to membrane trafficking in growing pollen tubes. However, evidence is needed to substantiate this, as signalling-dependent accumulation of DAG has not been detected in plant cells other than tobacco pollen tubes (Helling et al. 2006; Kost 2008).

ROP GTPases can transduce signals to downstream effectors, such as RICs (ROP-interactive CRIB motif-containing proteins), to implement cellular processes through divergent pathways. In pollen tubes, ROP GTPases can regulate actin

dynamics and tip growth via two counteracting downstream pathways. AtROP1 appears to operate downstream networks through two different RICs, RIC3 and RIC4. RIC3 participates in Ca^{2+} regulation, whereas RIC4 modulates the actin cytoskeleton. AtROP1 not only activates RIC3 to induce a high Ca^{2+} concentration in the apical region and actin disassembly, but also promotes RIC4 to stimulate actin polymerization and dynamics in the pollen tube (Gu et al. 2005; Lee et al. 2008). The apical accumulation of exocytotic vesicles oscillates in phase with, but slightly behind, apical actin assembly, and is enhanced by overexpression of *RIC4*, indicating a close link among RICs, exocytosis and actin dynamics in pollen tubes. Furthermore, *RIC4* overexpression inhibits exocytosis. This inhibition can be suppressed by Lat-B or RIC3, implying that polar vesicle accumulation depends on RIC4-mediated actin assembly, whereas the RIC3-dependent Ca^{2+} signal reduces vesicle accumulation at the tip, resulting in actin disassembly (Lee et al. 2008). It is apparent that the ability of ROPs to integrate counteracting pathways facilitates the fine tuning of actin dynamics during tip growth (Gu et al. 2005; Nibau et al. 2006).

In addition to the downstream effectors, ROP/RAC GTPases can also activate a signalling pathway that acts directly on the actin cytoskeleton by regulating the activity of ABPs (Bamburg 1999; Lawler 1999). Among the multiple mechanisms regulating actin binding and depolymerizing activity of ADFs/coffilins, Rho GTPases play an important role. For example, phosphorylation of NtADF1 at the conserved Ser-6 position is mediated by either NtRac1 in tobacco pollen tubes (Chen and Citovsky 2003) or a Ca^{2+}-dependent protein (Allwood et al. 2001). These results indicate that the interplay among ROP, Ca^{2+} signalling and the cytoskeleton is a complex network in pollen tube growth.

Compared to ROP downstream signalling pathways, knowledge of signalling upstream of the ROP small GTPases has been limited. Recent work in tomato suggests that a membrane receptor-mediated signalling may be upstream of the ROP pathway. LePRK1 and LePRK2 are leucine repeat (LRR)-containing membrane receptor-like kinases present in tomato pollen tubes. The extracelular LRR domain of LePRK2 can interact with the pollen protein LAT52 prior to germination, and switches to interact with a stigma-secreted protein, LeSTIG1, during and after germination (Tang et al. 2002, 2004). Pollen with reduced expression of *LAT52* failed to hydrate and germinate in vitro, and formed aberrant pollen tubes in the pistil (Muschietti et al. 1994). Knockdown of *LePRK2* has been shown to reduce pollen germination and tube growth rate (Zhang et al. 2008), suggesting that LePRK2 positively affects pollen germination and tube growth. The intracellular kinase domain interacts with KPP, a RopGEF, which plays a role in the polarity of the tube (Kaothien et al. 2005). These observations appear to be in line with the results showing that co-expressing *AtRopGEF12* and *AtPRK2a* (a *LePRK2* homolog in Arabidopsis) resulted in wider pollen tube tips (Zhang and McCormick 2007). Pollen tubes with reduced *LePRK2* expression display abnormal vacuole behaviour and impaired Ca^{2+} response (Zhang et al. 2008). These results indicate that the membrane receptor-like kinase LePRK2 most likely acts upstream of ROP signalling in controlling polar growth of the pollen tube.

13.4.5 Pectin Methyltransferase and Pectin Modification

The plant cell wall is a refined network of hemicelluloses and pectin, with cellulose microfibrils embedded in this matrix (Denès et al. 2000). In most plant species, the pollen tube cell wall consists of two distinct layers. The inner layer, composed of callose and cellulose, is a secondary wall that is first visible some distance behind the tip (15 μm in tobacco pollen tubes), while the outer wall layer contains mainly pectin and lacks cellulose (Ferguson et al. 1998). Unlike other plant cell walls, the components of the pollen tube tip comprise the outer wall layer, a single layer of pectin but not including callose or cellulose. For rapidly elongating pollen tubes, pectin is an extension-controlling cell wall molecule. Hence, the metabolism and modification of pectin are critical for pollen tube extension in the pistil. The pectin network is thought to be established chemically with three covalently linked components, namely homogalacturonan (HGA), rhamnogalacturonan-I and rhamnogalacturonan II (O'Neill et al. 1990). HGAs are linear polymers composed of 1,4-α-D-galacturonic acid (GalA) residues. GalAs represent the major component of pectin. These polysaccharides are synthesized, polymerized and methylesterified with side chains within the Golgi apparatus and, subsequently, transported via the Golgi-derived vesicles to the pollen tip and secreted into the wall in a highly methylesterified state (Sterling et al. 2001). Following vesicle discharge, HGAs are gradually de-esterified by wall-associated pectin methylesterases (PMEs; EC 3.1.1.11). During the process of de-esterification, the methoxyl groups of the polygalacturonic acid chain are converted into carboxyl groups. Thus, the acidic residues are exposed and cross-linked by Ca^{2+}, generating a new layer of pectin (Catoire et al. 1998; Limberg et al. 2000). The cell wall tends to stiffen, as a result of PME action and cross-linking by calcium. However, the activity of PMEs is influenced by the surrounding conditions. The localized reduction in pH, caused by protons produced in a de-esterification reaction, can inhibit some PME isoforms. It also stimulates the activity of cell wall hydrolases, and results in cell wall loosening, promoting wall yielding and cell expansion (Wen et al. 1999; Ren and Kermode 2000). Also, polar growth of the pollen tube tip is fine-tuned by the equilibrium of the interplay between these counteracting effects (Röckel et al. 2008).

In plants, PMEs represent a large family of enzymes that are involved in cell wall extension, cell adhesion, fruit maturation and senescence, cambial cell differentiation, and resistance to biotic attack (Tieman and Handa 1994; Wen et al. 1999; Micheli et al. 2000; Chen and Citovsky 2003). The Arabidopsis genome contains 67 PME-related genes, among which at least 18 isoforms are highly expressed in pollen (Pina et al. 2005), underlying their importance during pollen germination and/or pollen tube growth. PMEs are encoded mostly as pre-proproteins with a catalytic domain and an inhibitor domain (PMEI; Markovic and Jornvall 1992; Tian et al. 2006). The pre-region is required for protein targeting to the endoplasmic reticulum (ER), while the PMEI domain, also known as pro-region, is a large peptide of about 250 amino acid residues (Giovane et al. 2004). This peptide

regulates the correct folding of PME or inhibition of the enzyme activity to prevent premature demethylesterification of pectin (Micheli 2001).

Pollen-specific PMEs have been identified from a number of plant species, including willow (Futamura et al. 2000), tobacco (Bosch et al. 2005), maize (Wakeley et al. 1998) and Arabidopsis (Bosch et al. 2005; Jiang et al. 2005; Bosch and Hepler 2006; Tian et al. 2006). The loss of function of the pollen PME encoded by *VANGARD1* (*VGD1*) resulted in male sterility. Although *vgd1* mutant pollen grains are capable of germinating, pollen tube growth in the transmitting tract of the style is greatly retarded. Pollen tubes of *vgd1* frequently burst when germinated and grown in vitro. Further analysis indicates that the total pectin demethylesterification activity of PMEs in the *vgd1* pollen grains is decreased to 82% of that of the wild-type pollen. These results suggest that the *vgd1* mutation reduces PME activity in pollen grains, thereby altering the mechanical properties of the cell wall (Jiang et al. 2005). The second PME, AtPPM1 (*A. thaliana* pollen-specific PME1), is also highly and specifically expressed in Arabidopsis pollen grains. Compared with the wild-type pollen, overall PME activity is reduced by 20% in *atppm1* pollen grains. Mutation of *atppm1* has no apparent effect on pollen morphology, including the cell wall pattern. However, the pollen tubes exhibited curved, irregular morphology and are markedly stunted 6 h after germination, and the mutant pollen tubes remain short 24 h after germination (Tian et al. 2006). These results demonstrate the indispensable functions of PMEs in tip growth of pollen tubes. Consistently, application of exogenous PME causes a dramatic decrease in pollen germination rate and growth rate of pollen tubes in tobacco and lily. PME-treated pollen tubes of both species display a significant increase in cell wall thickness, especially at the apex (Bosch et al. 2005), suggesting that excessive PME is also detrimental to the pollen tube. It is therefore speculated that PME activity may be modulated by its inhibitors for pollen tube growth in vivo.

PEMIs, one of the most potent inhibitors of PME activity in vitro or in planta, have been identified (Wolf et al. 2003; Giovane et al. 2004; Raiola et al. 2004; Lionetti et al. 2007; Pelloux et al. 2007). Homology search reveals that there are at least 14 genes in Arabidopsis that may encode either PMEIs or invertase inhibitors (Rausch and Greiner 2004). Inhibition occurs through the formation of a reversible 1:1 complex of PME and PMEI (D'Avino et al. 2003). In the complex, PMEI almost completely covers the pectin-binding cleft that contains the active site of the PME (Johansson et al. 2002). The model of interaction between PME and PMEI may reflect the intramolecular binding of the PMEI pro-region to the PME domain (Hothorn et al. 2004). AtPMEI1 and AtPMEI2 are two proteinaceous inhibitors of PME specifically expressed in Arabidopsis pollen. They interact physically with each other, and AtPMEI2 inactivates AtPPME1 in vitro. While AtPMEI2 shows the growth-promoting effect, AtPPME1 exerts the growth-inhibiting effect on pollen tubes. The antagonistic effects of AtPMEI2 and AtPPME1 may correlate with their location in pollen tubes. In tobacco, AtPPME1-YFP is uniformly distributed throughout the cell wall of pollen tubes, including the tip region, but AtPMEI2-YFP is detected exclusively at the cell wall of pollen tube tips. Experimental evidence shows that AtPMEI2, but not AtPPME1, undergoes endocytic trafficking.

AtPMEI2 may be constantly and selectively internalized in the region 5–15 μm behind the tip, and accumulates exclusively in the apex to restrain the premature activity of freshly secreted PMEs from the Golgi apparatus (Röckel et al. 2008). These results show that polar distribution of PMEI isoforms in pollen tubes regulates cell wall stability via interaction with PMEs. However, it is not clear as to what mediates the formation and dissociation of the PME-PMEI complex, although the stability of the complex is influenced by pH (Giovane et al. 2004), and how this complex functions during tip growth of pollen tubes remains to be elucidated.

13.4.6 Pollen Tube Guidance

Unlike other polar growing cells, pollen tube growth is a guided process. Following germination on the stigma surface, pollen tubes penetrate the style and navigate through the transmitting tract, emerge from the septum, grow along the funicular surface and target precisely the embryo sac embedded in the ovule. Communication between the male and female is essential during this process. The whole process of pollen tube guidance is often divided into two phases, namely the sporophytic phase, which includes the stage when the pollen tube elongates through the transmitting tract of the pistil, and the gametophytic phase that includes the pollen tube emerging from the transmitting tract, growing along the funiculus, until its entry into the micropyle of the ovule (Higashiyama 2002; Y.H. Chen et al. 2007). How the pollen tube finds its way to the ovule is a question that has fascinated biologists for centuries (Lord 2003). However, the mechanisms underlying the pollen tube guidance to the ovule are being revealed. Chemoattractants for the pollen tube have been proposed. The presumed attractant is expected to be able to diffuse and form a concentration gradient that can lead to the directional growth of the pollen tube.

Multiple signals have been implicated to play a role in the sporophytic phase of pollen tube guidance. On the stigma surface, several potential cues have been proposed to direct pollen growth. These include lipids, water, ions and proteins. In the family Solanaceae, pollen germination and directional growth require either triglycerides in the stigma exudates, or a gradient of water established by the stigmatic lipids over an aqueous component of the exudates (Wolters-Arts et al. 1998). In the transmitting tract, several extracellular matrix proteins have been implicated in pollen tube guidance. The major extracellular arabinogalactans include TTS1 and TTS2 proteins in tobacco (Wang et al. 1993; Cheung et al. 1995; Wu et al. 2000), a 9-kd stigma-style cysteine-rich adhesion (SCA) protein (Park et al. 2000) and stylar pectic polysaccharides (Mollet et al. 2000) in lily and γ-amino butyric acid (GABA; Palanivelu et al. 2003). These substances provide nutritional and guidance cues, and support directional pollen tube growth in the transmitting tract of the female reproductive organ.

Guided pollen tube growth requires the presence of directional cues in the stylar tissue of the pistil. A simple model to achieve the directional growth is to form a gradient of a diffusible compound that acts as a chemotactic attractant for the pollen tube. This model requires a gradient of attracting signal in the female sporophyte and a sensing system in the pollen tube. It has been suggested that TTS glycoproteins may serve as guiding cues in the pistil (Cheung et al. 1995; Wu et al. 1995, 2000). These glycoproteins bind to the pollen tube surface and are incorporated into the pollen tube wall in the process of tube elongation (Wu et al. 2000). Also, TTS proteins possess features of positive chemotropic signals, for example, attracting pollen tubes, promoting tube elongation when applied in vitro, slowing the pollen tube growth in vivo by reduction of TTS levels, and the ability to form a gradient of glycosylation levels in the transmitting tissue (Cheung et al. 1995; Wu et al. 1995, 2000). In addition, a haptotactic model has been proposed by analogy to neuron guidance in animals, where a large extracellular matrix molecule (laminin) forming a trail, and a small signalling peptide (netrin) sensed by a neuronal receptor act together to guide neuron growth. In agreement with such a model, it has been shown that a large matrix molecule (pectin) and a small peptide (SCA) together are responsible for pollen tube adhesion in the lily style (Lord 2003). This finding suggests that both haptotactic and chemotactic mechanisms might be involved in pollen tube guidance in the style. However, it is not clear whether haptotactic mechanisms are unique for open styles and chemotactic mechanisms for solid styles, as in Arabidopsis.

Studies on GABA action in pollen tube guidance support the view that the gradient of GABA is critical for normal growth and guidance of pollen tubes in female tissues (Palanivelu et al. 2003). GABA is a four-carbon ω-amino acid previously known as an inhibitory neurotransmitter, and plays roles in developing nervous systems in animals (Petroff 2002). In Arabidopsis, the formation of an increasing gradient of GABA from stigma to the micropyle of the ovule by POLLEN PISTIL IINTERACTION 2 (POP2) has been proposed. *POP2* encodes a transaminase that degrades GABA. Compared to wild-type tissues, the recessive *pop2* mutation results in more than a 100-fold increase in GABA levels in mutant tissues. The increase in GABA concentrations and elimination of a gradient offer an explanation to growth arrest and loss of micropylar pollen tube guidance observed in the *pop2* mutant. However, the *pop2* mutant is male and female fertile when crossed with the wild type. Furthermore, pollen tubes of the *pop2* mutant, but not the wild type, fail to target the *pop2* mutant ovule, suggesting that *POP2* tubes may turn over GABA efficiently. However, *pop2* mutant tubes are hypersensitive to exogenous GABA, presumably because they lack the enzyme that degrades it (Palanivelu et al. 2003). In animals, GABA interacts with its receptors through different mechanisms to function as an inhibitory neurotransmitter in the central nervous system (Pinal and Tobin 1998; Satin and Kinard 1998; Brice et al. 2002). Similar receptors have not been identified in plants. In addition, pollen tube guidance is a species-specific feature. It remains to be seen how GABA, as an attracting signal, fulfils such a requirement.

Another possible attracting signal is the maize EGG APPARATUS 1 (ZmEA1) protein (Márton et al. 2005). Knockdown of *ZmEA1* expression by RNAi technique abolishes micropylar pollen tube guidance in maize. The *ZmEA1* gene encodes a small secreted protein and is expressed in the egg apparatus of the embryo sac. Bioinformatics analysis has identified ZmEA1 homologues in other plant species, including Arabidopsis and rice (Gray-Mitsumune and Matton 2006). The EA1 proteins share a conserved 27–29 amino acid motif (EA box) near the C terminus and are divergent outside the box. This satisfies the requirement for species specificity as a signalling molecule. However, it is not clear whether EA1-like proteins can also function in pollen tube guidance in other species.

Genetic and laser ablation studies demonstrate an essential role of the gametophytic cells in pollen tube guidance. Laser ablation of the synergid cell, but not the egg and central cell, abolishes micropylar pollen tube guidance in *Torenia*, which is characterized by the embryo sac that protrudes from the ovule integuments and is accessible to experimental manipulation (Higashiyama et al. 2001). The two synergid cells act synergistically, since ablation of one synergid reduces the efficiency of pollen tube guidance. In the Arabidopsis mutant *eostre*, although the gametophytic cells of the embryo sac are mis-specified, resulting in two egg cells and only one synergid cell, pollen tube attraction takes place normally and sperm cells are released correctly in some mutant embryo sacs (Pagnussat et al. 2007). In most species, the pollen tube enters the synergid cell by extending through the filiform apparatus, a thickened cell wall extension of the synergid cell at the micropyle. Loss-of-function mutation of the synergid-expressed gene *AtMYB98* in Arabidopsis causes a defect in the filiform apparatus and pollen tube guidance (Kasahara et al. 2005). As discussed above, knockdown of the egg/synergid-expressed *ZmEA1* in maize also leads to defective pollen tube guidance (Márton et al. 2005). Taken together, these results suggest an essential role of the synergid cell in pollen tube guidance.

In addition to the synergid, other gametophytic cells of the embryo sac, such as the egg and central cell, may also play a role in pollen tube guidance. In Arabidopsis, mutation in the central cell-expressed gene *AtCCG* (*CENTRAL CELL GUIDANCE*) abolishes micropylar pollen tube guidance, demonstrating a critical role of the central cell in guidance (Y.H. Chen et al. 2007). *CCG* encodes a nuclear protein that most likely acts as a transcription regulator in Arabidopsis. Pollen tubes cease to grow near the micropyle, or turn away or bypass the micropyle of the mutant embryo sacs, indicating that the mutant embryo sacs are unable to produce or emit the attracting signal. Furthermore, mutation of the egg-expressed gene *GEX3* also abolishes pollen tube guidance in the ovule (Alandete-Saez et al. 2008). *GEX3* is expressed in the egg cell and pollen grains, and it encodes a plasma membrane-localized protein of unknown function. These findings indicate that pollen tube guidance is the integrative effects of multiple signalling pathways and combined contribution of individual gametophytic cells. Compared to the female guidance during the tube elongation, little is known about the mechanism of how pollen tubes perceive and respond to the female attracting signals.

13.5 Conclusions

As a tricellular free-living male germ unit, pollen possesses many unique features, such as its specialized cell wall, and has been the focus of developmental genetics research in recent years. The pollen tube has been used as a model for understanding polar cell growth for many years. Pollen germination and tube growth are the results of orchestrated interactions among diverse signalling pathways (Ca^{2+} and others), small GTPases and the cytoskeleton. Although a large volume of information has been gained, especially in pollen tube cell biology in past decades, molecular mechanisms governing pollen germination and tube growth remain to be elucidated in the context of modern developmental genetics. More recently, pollen transcriptome and proteome analyses have generated tremendous amounts of data. If combined with large-scale genetic and biochemical analyses, these data may help to provide insights into the regulatory mechanisms of pollen germination and tube growth.

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Chapter 14
Fertilization in Angiosperms

S.D. Russell

14.1 Introduction

In the current review, focus is directed to the most recent findings, with an attempt to integrate critical information that is useful in understanding the current biological context of double fertilization research in angiosperms. An attempt has been made (insomuch as is possible) to provide a new synthesis, but some overlap is inevitable. Double fertilization is a key topic of interest in plant reproduction, and key molecules and mechanisms are primed for significant breakthroughs within the near future (Berger 2008). A recent special issue of *Sexual Plant Reproduction* (Russell and Dresselhaus 2008), entitled “Deciphering molecular mechanisms of fertilization in seed plants”, describes the state of the art within the context of myriad topics centered on fertilization, including pollen tube attraction (Higashiyama and Hamamura 2008), synergid function (Punwani and Drews 2008), control of male germ lineage expression (Singh et al. 2008), molecular fertilization molecules in plants as compared to animals (Márton and Dresselhaus 2008), experimental manipulations using in vitro fertilization (Kranz and Scholten 2008), mechanisms preventing polyspermy (Spielman and Scott 2008), and activation of development (Curtis and Grossniklaus 2008). Past reviews in this decade summarize the history of double fertilization (Raghavan 2003), and provide coverage to the first half of this decade (Lord and Russell 2002; Weterings and Russell 2004; Wang et al. 2006). In the current review, some of the most important topics are highlighted without overlapping other readily available reviews.

S.D. Russell
Department of Botany and Microbiology, University of Oklahoma, Norman OK 73019, USA
e-mail: srussell@ou.edu

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14.2 Angiosperm Reproduction: a Matter of Structure, Timing, and Physiology

Angiosperms, as a most diverse phylum of higher plants, display great diversity in fertilization events amongst higher organisms. This diversity is reflected in differences in structural organization that are well described in past reviews (e.g., megagametophytes, Huang and Russell 1992), but also in the timing of various maturational cues. Such timing differences may slow or speed the position that pollination plays in the process and the duration of the progamic phase (from pollination to fertilization), and also extend to receptivity of male and female gametes. Fertilization involves a precise choreography of signals, and is based on communication signals that arise from both the male and female gametophyte that synchronize receptivity, as well as timing cues from the gynoecial tissues of the maternal sporophyte (Dresselhaus 2006). In fact, disruption of this timing through heterochronic shifts appears to be a common theme of disruptions of sexual reproduction, such as triggering apomictic reproduction (Bradley et al. 2007).

14.3 Pollen Biology and Maturation

Pollen is frequently disseminated in an immature state, and may require the formation of sperm cells after pollination, and in many cases cell cycle advancement as well, to become mature. The need to coordinate female and male maturity to optimize receptivity and success in fertilization is one reason for a diversity of timing strategies in angiosperms. There is, for example, no reason for precocious pollen development in orchids, as ovules are not even formed until successful pollination occurs. Male and female choreography of maturation is also evident even near the moment of fertilization, as in male gametes of tobacco, where cell cycle changes occur even in the synergids (Tian et al. 2005). It should not be surprising that angiosperms, which are at an apparent peak in their morphological diversity, would display considerable diversity in reproductive biology that extends to the gamete level.

14.3.1 Bicellular Versus Tricellular Pollen

A most obvious distinction in developmental synchronization is whether the pollen grains are disseminated in the bicellular condition (containing the vegetative and generative cells) or the tricellular condition (containing vegetative cell and two formed sperm cells). Timing of sperm formation relative to anthesis is associated not just with male gamete maturity, but with a suite of physiological characteristics that determine numerous features of pollen development that involve the vegetative cell, including recalcitrance for grains to germinate, tube elongation rate, incompatibility responses, and classes of self-incompatibility. Bicellular pollen is typical

of basal angiosperms, found in nearly two-thirds of the angiosperms, and thus is usually a plesiomorphic condition. In rare cases, however, this characteristic may have been derived secondarily for better timing with female maturation. Tricellular pollen, in contrast, is typically an apomorphic character for angiosperms, but interestingly the precocious formation of sperm cells may still be accompanied by many hours of pollen tube elongation before fusion, as a formed sperm cell is not necessarily a mature or receptive sperm cell.

In bicellular pollen, fertilization is delayed until sperm cells are formed, which usually occurs after several hours of pollen tube growth in the style; mitotic division of the generative cell occurs within the narrow confines of the pollen tube. The two-phase pattern of elongation characteristic of bicellular pollen—initially slower elongation with relatively broad nutritional needs, followed later by rapid elongation with complex nutritional requirements after sperm cell formation—suggests a significant shift in gene expression in the male gametophyte that accompanies sperm cell formation. Tricellular pollen, in contrast, tends to have demanding requirements for germination from anthesis, and some cannot tolerate water at all except in a bound form. Elongation of pollen tubes in vitro may be more closely mimicked by culturing grains on semi-solidified agar or agarose to provide a semi-solid substrate for elongation. Under ideal conditions, pollen tube elongation in vitro may approach the normal life span and elongation range of in vivo pollen tubes (Boavida and McCormick 2007).

Accompanying bicellular and tricellular expression, there may also be shifts in regulation of genes in the male germ lineage (Singh et al. 2008). Interestingly, promoters of bicellular pollen tend to retain complete functionality in bicellular and tricellular pollen, but tricellular male gamete promoters introduced into ancestral bicellular plants are unpredictably expressed (Gou and Russell, unpublished data). Rarely in flowering plants are both bicellular and tricellular pollen produced in the same plant, the most conspicuous being *Populus*. It is tempting to speculate that studying expression in such a genomic model may provide insight on how timing of sperm cell formation may be controlled, and the means by which two types of pollen may even coincide within a single anther.

14.3.2 Pollen and Sperm Maturation, Cell Cycle, and Cellular Identity

The completion of sperm maturation is independent of sperm cell formation; sperm cells are not mature at inception, but acquire maturity by conditioning cell surface changes and cell cycle changes (Rotman et al. 2005). For cells to fuse successfully requires meeting all necessary conditions at the same time. In tobacco, for example, newly formed male gametes can be fused easily by simply placing them in apposition for a brief period (Tian and Russell 1998). As the sperm cells mature, their cell surfaces become dramatically modified and more resistant to indiscriminant fusion throughout the first 10 h after their formation. Although some success has been

obtained in isolating sperm cells from bicellular pollen using in vitro techniques alone, a more consistent protocol is the in vivo/in vitro culture method. Briefly, an intact stigma is pollinated, and then the style is cut and floated vertically on a liquid medium. Once tubes grow out of the cut style, male germ cells are collected. Although tobacco male gametes appear to remain wall-less, changes are evident in their periplasmic surface, because incidental fusion does not occur. These modifications can be reversed by the use of dilute wall-digesting enzymes; interestingly, both pectinase and cellulase are required in such older sperm cells, and once treated, cells fuse readily (Tian and Russell 1998).

Another maturational change is that pollen tubes need to contact the gynoecial matrix to acquire the ability to navigate to the ovule. According to Higashiyama and Hamamura (2008), *Torenia* pollen tubes and those of other species are insensitive to directional signals emanating from the ovule, unless the pollen is allowed to elongate on the stylar tissues. Once subjected to the gynoecium, pollen tube elongation becomes strongly directional, and tip growth is readily reoriented if it is within the sphere of its ability to detect the signal (see Higashiyama and Hamamura 2008, online supplementary video S2).

Male and female gametes must reach synchrony in the cell cycle at either G1 or G2, depending on the receptive stage required for fertilization in that species. In animals, with few exceptions, this is the G1 stage of the cell cycle, but in flowering plants gametes may fuse at either the G1 or G2 stage (Friedman 1999). Sadly, only few studies report this important behavior, needed to predict the likelihood of success in fusing flowering plant gametes in vitro. The effect of cell cycle incongruity is dramatic (Wang et al. 2006), as fused male and female gametes that are not at the correct stage result in fusion products that never divide, and become arrested in development. Tobacco provides an illustrative example, as it is a model for ease of plant regeneration, yet in vitro combinations failed to divide (Tian and Russell 1997). As later research clearly showed, bicellular pollen readily produced sperm cells that remained in G1 throughout stylar passage, but at seemingly the last possible moment before fusion, within the degenerated synergid, male gametes completed S phase and fused with female gametes at G2 phase (Tian et al. 2005). The presence of male gametes in the synergid, in turn, appeared to stimulate precocious entry of female gametes into G2, thus synchronizing their cell cycles and removing the block. There are numerous patterns reported for cell cycle condition during fertilization, and charts displaying all of the reported patterns are included in Friedman (1999) and Tian et al. (2005).

Although animal cells have persistent germ cell lineages that involve significant complexity in targeting and timing, flowering plants appear to be able to generate germ cell lineages through principally positional cues. These seem to be initiated spontaneously, but still the gametes represent a “stem” cell lineage in the sense of being a founder lineage for further development. Although they do not possess a permanent germ cell lineage in the same manner as animals, their condition is critical to successful function. Positional cues may also be involved in controlling apomixis, and in returning apomictic plants to sexual reproduction as well (Okada et al. 2007).

Male gametes appear to acquire identity through CDKA;1, a cdc2 homolog that is required for fertility. In *Arabidopsis*, loss-of-function *cdka-1* mutants produce bicellular pollen grains that do not progress to sperm cell formation. Interestingly, the generative cell may behave as if it were a sperm cell and fertilize the egg cell, and in this fusion it appears to fuse preferentially with the egg cell, rather than the central cell (Iwakawa et al. 2006). Fertilized products, however, typically fail to grow beyond the 8-celled stage, because of endosperm failure. Yet, when Nowack et al. (2006) crossed *cdka-1* sperm cells into a *fis* homozygous female mutant, the *cdka-1* embryo was rescued by the fertilization-independent endosperm background. Another mutant, *duo*, which has an altered MYB transcription factor, also arrests generative cell division, but the *duo* generative cell reaches 2.6C and its unicellular "hypersperm" does not fuse (Rotman et al. 2005; Twell 2006). A third generative cell arrest mutant, *msi1*, has altered function of Chromatin Assembly Factor 1 (CAF1) that may produce defects also mimicking endoreduplication, as result of a failure to target acetylated histone H3-H4 dimers to newly synthesized DNA (Chen et al. 2008). The *msi1* mutants successfully fuse, and in doing so fuse in essentially equal proportions between the egg and central cells (Chen et al. 2008). Further examination of sperm fusions in the *cdka-1* and other lines may provide critical information about whether there is an inborn differential capacity of sperm cells to undergo double fertilization.

14.3.3 Attraction of Pollen Tubes to the Female Gametophyte

As indicated by synergid ablation experiments, the attraction of pollen tubes to the embryo sac clearly resides in the persistent synergid. Neither egg cells nor any other existing cells could attract the tube according to the experiments of Higashiyama et al. (2001), and the attraction is preferential at the species level (Higashiyama et al. 2006). The attracting molecules may, in fact, be quite distinct for monocotyledons and dicotyledons. The precise identity of attractant molecules is limited for the most part to EA1 in maize (Márton et al. 2005), but undoubtedly this number will grow. Needless to say, when *EA1* or other genes coding for synergid attractors lose function, fertilization is no longer a likely outcome. A central cell attraction-related apparent transcription factor encoded by *CCG* has been discovered in *Arabidopsis* ovules that, if inactivated, no longer attracts pollen tubes to the embryo sac (Chen et al. 2007). Although the exact mode of action is not known, the protein is believed to be restricted to the central cell, and likely the nucleus. Presumably, it acts upstream of other elements that are mobile, and may provide a key for identifying these molecules.

The nature of attraction of the synergids is the topic of a number of current studies. A remarkable video of short-distance attraction by pollen tubes to a synergid is available online (Higashiyama and Hamamura 2008, supplementary video S2), showing the rapidity of tube reorientation possible in response to ovular signaling. Also noteworthy is that such molecular attraction appears to cease after successful penetration, and that fertilized ovules may actually repel approaching pollen tubes (Fig. 14.1; Palanivelu and Preuss 2006).

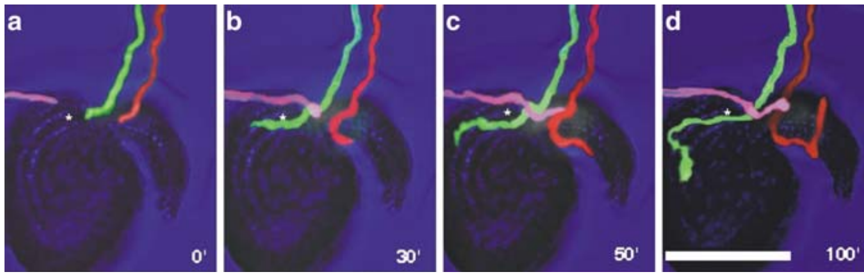

Fig. 14.1 Attraction to unfertilized ovules occurs prior to fertilization, followed by later repulsion. **a–d** Time series (in min) of pollen tube interactions. The *center* tube entered the micropyle (*), whereas the *left* and *right* tubes arrived after fertilization. Scale bar, 100 μm. (Modified from Palanivelu and Preuss 2006)

The nature of the exudate that generates the attractive signal is located downstream of synergid transcription factor MYB98 (Kasahara et al. 2005), which is known to control positively a battery of synergid genes that are involved in tube attraction. In total, MYB98 controls at least 83 downstream genes, including 48 genes within four gene families that encode Cys-rich proteins, and is one branch of a multi-tiered gene regulatory network (Punwani et al. 2008). Among the specific products secreted according to one screen are nine expressed proteins that included a hypothetical protein, a polygalacturonase, galactosyltransferase, a rapid alkalinization factor family protein, a putative annexin, plastocyanin-like domain-containing protein, and a subtilase family protein (Punwani et al. 2007). A screening of transcripts showing altered expression in a differential screening of *myb98* mutant ovules using an *Arabidopsis* tiling array indicated 77 downregulated and 40 upregulated genes compared to wild type (Jones-Rhoades et al. 2007). These products included some 22 newly identified genes; among the protein domain groupings noted, products included DUF784, DUF1278, PSIL (*Papaver* self-incompatibility-like), DEFL (defensin-like), DUF239, subtilisin, PMEI, glycosyl hydrolase 17, and GDSL lipase motifs (Fig. 14.2). Interestingly, a proteomic screen of pollination droplets of four disparate gymnosperms found proteins that include subtilisin-like proteinases, glycosyl hydrolase, glucan 1,3-β-glucosidase and precursor, β-D-glucan exohydrolase, glucanase-like protein, thaumatin-like proteins, and chitinase motifs (Wagner et al. 2007), and these appear comparable to proteins secreted by synergids. Likely, there are elements of common solutions as well as a likelihood of common descent in these secretions, despite their different origins.

14.4 Fertilization: Receipt of Pollen Tube and Plasmogamy

Although a persistent synergid is the source of attractive signals, the degenerate synergid is the one that receives the pollen tube and its contents. The synergid may degenerate before fertilization through a process of programmed cell death

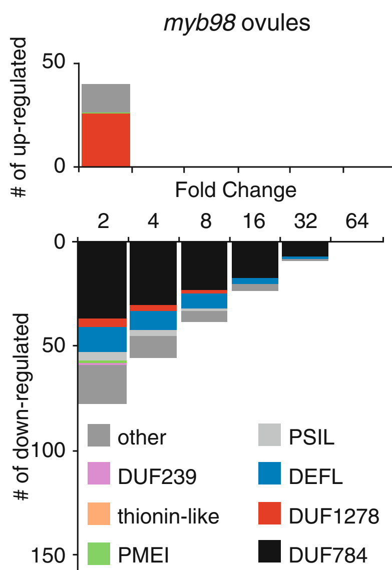

Fig. 14.2 Differentially expressed families of small, secreted proteins in *myb98* ovules, upregulated with respect to wild type (*above*, from top: other & DUF1278) and downregulated (*below*, from top: DUF784, DUF1278, DEFL (defensin-like), PSIL (*Papaver* self-incompatibility-like), PMEI (pectin methylesterase inhibitor), DUF239, other). Number of genes differentially expressed ($p < 0.001$) at indicated cutoffs (>2-fold, >4-fold, >8-fold, >16-fold, >32-fold, and >64-fold). (Modified from Jones-Rhoades et al. 2007)

(An and You 2004). In *Arabidopsis*, degeneration appears to be triggered directly by physical interaction with the pollen tube that does not appear to precede tube arrival (Sandaklie-Nikolova et al. 2007). Alternatively, degeneration may occur upon direct entry of the pollen tube (Weterings and Russell 2004).

The degeneration of the receptive synergid appears to assist fertilization by presenting the pollen tube with an embryo sac location that facilitates (i.e., is less resistant to) penetration. Pollen tube discharge into the synergid clearly indicates entry into a specific micropylar site, followed promptly by formation of an aperture in the tube, and discharge of the tube's contents (Higashiyama et al. 2000, and online supplementary video). This positional context also provides access specifically to a region within the embryo sac that is suited to fertilization, guiding sperm cells to a region of the egg and central cell, where cell walls are lacking and plasma membranes of the female gametes are conducive to plasmogamy. This is a relatively small region, where sperm cells often may contact with both female gametes accompanying adherence (Russell 1992). Reorganization of the cytoskeleton of the prior synergid may also aid in the process of gamete migration to the sperm cells' respective fusion sites (Weterings and Russell 2004). Involvement of an actin template in the receptive synergid, and spontaneous association of pollen myosin with the sperm surface may aid in conveying male gametes to the fusion site. Upon plasmogamy, both male gametes fuse with a female gametic cell, forming the zygote from the fusion of egg cell and sperm, and the endosperm from the fusion of central cell and sperm. Nuclei migrate into apposition with female nuclei, and nuclear fusion ensues.

14.5 Female Gametophyte Cell Multiplication Control and Identity

A number of genetic screens have been conducted to search for fertilization defective genes, but there are many paths to failure, and only recently have candidates for control of female gamete identity emerged. Among these identity determining genes, the female gametophyte mutant *eostre* was shown to switch synergid function to that of an egg cell by misexpression of a BEL-like homeodomain gene (BLH1), which is not normally detected in wild-type ovules (Pagnussat et al. 2007). This misexpression triggered reorientation of synergid polarity, as well as defects in nuclear migration that seem to affect cell identity. Pagnussat et al. (2007) propose that cell determination is based primarily on positional cues, and this is supported as well by the long-known *indeterminate gametophyte* (*ig1*) mutant, which produces multiple egg cells, synergids, and polar nuclei. The production of this multiplication of female gametes also appears to stimulate multiple fertilization events, so these are properly identified cells (Guo et al. 2004). *IG1* was just recently found to encode a LATERAL ORGAN BOUNDARIES domain protein that also appears to trigger polarity defects during early development in leaves as well (Evans 2007). In sporophytes, this factor is noted to bear high similarity to *ASYMMETRIC LEAVES2* of *Arabidopsis*. Irregularities in female gametophytic cell formation are also evident in *gametophytic factor 1* (*gfa1*) mutants of *Arabidopsis*, possibly a downstream effect of a defective RNA splicing factor (Coury et al. 2007). Other pathways may also lead to numerical errors in embryo sac cell formation. After emergence of cell identity in two female gamete target cells, namely, the egg cell and central cell, their identity appears to be fixed with activation of LACHESIS (named after the Greek Moirae who drew lots to determine fates). Once LACHESIS expression is designated, female gametic identity is established, and other accessory cells of the female gametophyte are inhibited from assuming a gametic identity (Groß-Hardt et al. 2007). The stability with which these cells may be marked in basal angiosperm lineages, however, may still be subject to study, given the apparent liability of gametophyte organization in these plants (Williams and Friedman 2004; Friedman 2006).

Female gametophyte cellular differentiation has also been examined by comparing RNA generated by *dif1* mutants with wild-type ovules, using an Affymetrix ATH1 genome array. In the context of the ovule, 11 genes were expressed exclusively in the antipodal cells, 11 genes were expressed exclusively or predominantly in the central cell, 17 genes were expressed exclusively or predominantly in the synergid cells, one gene was expressed exclusively in the egg cell, and three genes were expressed strongly in multiple cells of the female gametophyte (Steffen et al. 2007). Using the Affymetrix 24K ATH1 microarray, an expanded survey of potential female gametophyte genes based on comparing wild-type ovules with another embryo sac-lacking mutation revealed 1,260 active embryo sac genes (Johnston et al. 2007). Based on these screens, additional female candidates involved with fertilization may ultimately be characterized further.

Arabinogalactans have long been suspected to play a role in cellular identity (Pennell and Roberts 1990; Southworth and Kwiatkowska 1996). The first direct functional evidence for the necessity of arabinogalactan proteins was discovered using an enhancer trap in immature *Arabidopsis* ovules (Acosta-Garcia and Vielle-Calzada 2004); plants with an impaired version of the gene failed to initiate female gametogenesis. Since this is a classical arabinogalactan, it contains a domain responsible for attaching the protein backbone to a glycosylphosphotidylinositol (GPI) membrane anchor. In functional assays, it appears that this AGP is integral for development. Coimbra et al. (2007) have provided additional evidence for the involvement of arabinogalactan protein in later ovules, localizing the monoclonal antibodies JIM8, JIM13, MAC207, and LM2 to arabinogalactan epitopes in secretory synergid cells, and along the pollen tube pathway into the ovule; in this case, the arabinogalactan proteins may simply be primarily for guidance of *Arabidopsis* pollen elongation in the pistil. Such surface label may be expected to provide spatial and possibly temporal cues for fertilization.

14.6 Cell Fusion Determinant GCS1/HAP2

A number of fertilization critical genes have been found, and these are currently being characterized. Among the most interesting are those discovered through screening of pollen tube guidance-affecting genes, initially called "hapless" for their lack of directionality (Johnson et al. 2004). Among these is a sperm-specific expressed gene that, when altered, inhibits gamete fusion in all cases, and is variable in its severity relative to pollen tube guidance (von Besser et al. 2006). Interestingly, Mori et al. (2006) reported independently on the discovery of GCS1 (Generative Cell Specific-1) from lily ESTs, and using its sequence identified a homolog in *Arabidopsis* that corresponded to the same locus as HAP2 (At4g11720), with wide sequence conservation among green plants and a number of other protists. GCS1/HAP2 appears to be a highly conserved membrane protein, as it is shared with the minus strain of *Chlamydomonas*, the mating stain that corresponds to male function in this unicellular green alga that appears to be basal to land plants. Further functional characterization of these molecules in both *Chlamydomonas* and *Plasmodium* gametes revealed that HAP2 is necessary for cell fusion, but not adhesion, at the membrane level, and is thus conserved in a non-photosynthetic protistan lineage of eukaryotes, as well as green plants (Liu et al. 2008). The mechanism of this highly conserved molecule may therefore uncover a long-sought molecular trigger for gamete fusion.

14.7 Fertilization Limiting Genes

Genetic dissection of function during fertilization has identified a number of critical fertility limiting genes. Among the most conspicuous of these mutants are those involved in synergid function. Screening in *Arabidopsis* has provided information

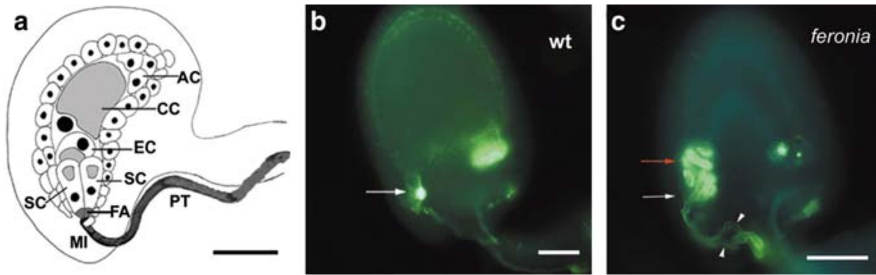

Fig. 14.3 Pollen tube reception mediated by the FERONIA/SIRÈNE receptor-like kinase. **a** Diagram of mature female gametophyte. Scale bar, 30 μm. **b**, **c** Aniline blue-labeled ovules, provide an epifluorescent label for pollen tube wall callose. **b** Fertilized wild-type ovule. *White arrow* Site of pollen tube arrest. **c** Unfertilized *fer* gametophyte in ovule. *White arrow* Site of pollen tube entry/arrest in wild-type ovules, *other arrow* continuing elongation of pollen tube in the female gametophyte, *arrowheads* multiple pollen tubes entering same ovule. Scale bars, 30 μm. Abbreviations: AC, antipodal cells; CC, central cell; EC, egg cell; FA, filiform apparatus; MI, micropyle; PT, pollen tube; SC, synergid cells. (Modified from Escobar-Restrepo et al. 2007)

on numerous defects leading to sterility (Christensen et al. 2002); one in particular showed an ability to attract pollen tubes, but was not able to transfer gametes. In *gametophytic factor 2* (*gfa2*), a mitochondrially expressed DnaJ chaperonin is impaired that apparently prevents programmed cell death in the synergid (but not antipodals). In these plants, the intact synergid fails to transmit male gametes to the female cells.

The time required between arrival and discharge of the pollen tube may be essentially immediate, as in *Torenia* (Higashiyama et al. 2000), whereas in other species, it is a much more controlled process. For example, in *Arabidopsis*, 2 to 3 min will elapse between pollen tube entry into the synergid and discharge (Rotman et al. 2003, and online supplementary video). Three mutants have been characterized in detail that appear to interfere with pollen tube discharge without impairing synergid degeneration (Fig. 14.3). In the *Arabidopsis* mutants *feronia* (*fer*; Huck et al. 2003), *sirène* (*srn*; Rotman et al. 2003) and *scylla* (*syl*; Rotman et al. 2008), there are various defective expressions of the *FERONIA/SIRÈNE* receptor-like kinase that have common expression in a synergid defect that prevents pollen tube rupture (Escobar-Restrepo et al. 2007). Synergid degeneration may be variable in these mutants, but these alleles all have in common that there is clear female control over the cessation and rupture of the pollen tube. The normal protein corresponds to a plasma membrane-localized receptor-like kinase that has been localized at the base of the synergid. Thus, it is evident that the process of normal pollen tube discharge is under the control of a number of molecules, and that these may each be involved in conditioning the transfer of the male gametes, clearly preventing transfer in the case of critical mutants.

Male determinants required for fertilization also occur. A pollen plasma membrane-bound Ca^{2+}-ATPase was demonstrated to be necessary for fertilization in *Arabidopsis* (Schiøtt et al. 2004). Knockout mutants showed two forms of impairment. Pollen tubes frequently did not reach their normal distance during

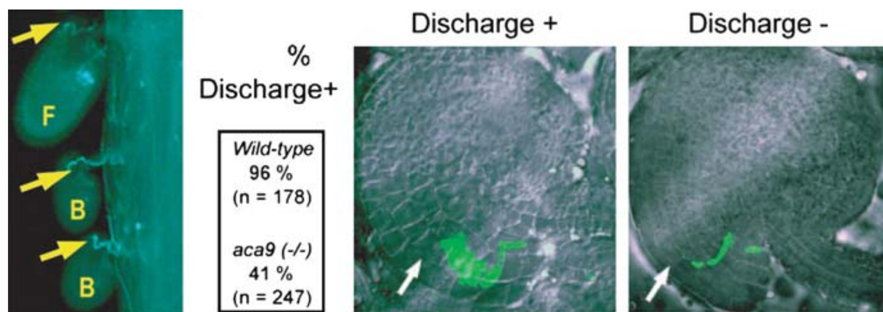

Fig. 14.4 *aca9* pollen tubes indicated blocked (B) or successfully fertilized (F) ovules (*arrows* pollen tube tip inside ovule). "Discharge +" image shows *ACA9* tubes, as viewed using a *ACA9*-promoter::GFP pollen cytoplasmic label. Note spread of fluorescent label, indicating successful penetration of degenerated synergid. "Discharge−" image shows GFP-labeled pollen cytoplasm retained in an intact tube, indicating aborted pollen tube discharge. (Modified after Schiøtt et al. 2004)

pollen tube elongation, and even if they successfully located and penetrated the synergid, the tube failed to discharge (Fig. 14.4). Another known mutant, called *abstinence by mutual consent* (*amc*), prevents pollen tubes from discharging only if absent from both male and female gametophyte (Boisson-Dernier et al. 2008). Although the mutant corresponds to an ortholog of a peroxin targeting protein (PEX13), there is question about its normal function and involvement, if any, in peroxidase activation and targeting, as is true in canonical PEX proteins. If it is involved in rupturing the pollen tube during male gamete release, then synergid and tube proteins are apparently functionally interchangeable, and proteins may even be expected to mix in the degenerated synergid. Interestingly, *WOX5*, a gene of the Wuschel homeobox family, is also required for pollen tube elongation in *Arabidopsis*, and may be involved with mediating auxin signaling in pollen (Dorantes-Acosta and Vielle-Calzada 2006). The earliest clear defect of normally viable pollen to date seems to be *pgd1* mutant, which is a T-DNA modified form of the *XLG1* gene (encoding an extra-large GTP-binding protein), resulting in failure to germinate (Ding et al. 2007).

14.8 Nuclear Fusion

The molecular basis of nuclear fusion is an obvious target for understanding karyogamy during fertilization. With this goal, Portereiko et al. (2006) screened developing embryo sacs of *Arabidopsis*, and found six nuclear fusion defective genes (*nfd1* to *nfd6*) that alter fusion of polar nuclei during megagametogenesis. Five of these represented complex failures that obscured assaying their ability to undergo karyogamy, but *nfd1* retained the capacity to undergo normal sexual reproduction, and showed inability to undergo karyogamy. The best-characterized mutant apparently alters *RPL21M*, which encodes a mitochondrial 50S ribosomal

protein known as L21. Its localization to the mitochondrion was confirmed by green fluorescent protein (GFP) protein fusions, and a wild-type copy of this gene rescued the mutant phenotype. That the karyogamy inhibition is downstream of L21 function suggests that some protein synthesized by the mitochondrion is critical to nuclear fusion of sperm, egg, and polar nuclei, but it does not clearly point to a candidate. Nuclear fusion is known to involve fusion of the outer nuclear envelope first, which originates from rough endoplasmic reticulum (Russell 1992). Although this endoplasmic reticulum bears ribosomes on its surface, it is possible that this alteration of L21 is incidental, as the ribosomes expressed in this region would likely be exclusively of nuclear/cytoplasmic origin.

14.9 Cytoplasmic Transmission in Gametes

Initially, exclusive use of Feulgen nuclear stains to visualize pollen tubes gave rise to a long-held misconception that sperm existed as “bare nuclei”, but careful examination of the male sperm cells revealed that all male gametes of angiosperms are truly cellular, and this is the basis of initial plasmogamy (fusion of male and female gamete cells) prior to karyogamy (Russell 1992). Thus, there is a potential basis for transmission of paternal DNA through cytoplasmic organelles (mitochondria and plastids), but their participation may be variable, based on cytoplasmic transmission during plasmogamy, quality and quantity of DNA transmitted, and other mechanisms, including cellular placement of organelles (Mogensen 1996). Reports of strictly maternal organellar inheritance dominate the literature, but the relatively common occurrence of heritable organelles in the male germ lineage calls into question whether this can be supported. Although measurable transmission of paternal organelles may be unimportant in most contexts—relative to the much stronger transmission of maternal organelles—the male input is certainly large enough to assure paternal transmission, and inheritance does occur on occasion.

Typically, paternal organelle transmission is unimportant, but it may become significant in the context of organellar DNA, particularly if this is to be part of a biocontainment mechanism to halt potential dissemination of novel genes. Clear evidence exists from developmental examination of generative and sperm cell organelles that the number of organelles is subject to a severe founder effect, as generative cells have few organelles initially, and after an initial proliferation of organelles, organelle numbers for mitochondria and plastids decline later during development (Russell and Strout 2005). Only one plant to date has been found to lack heritable organelles completely. Participation of male cytoplasm during plasmogamy may also be variable during fertilization. A number of patterns of male cytoplasmic transmission have been reported in the literature, most of which support male transmission to some extent, and others exclude or relegate cytoplasmic organelles to a terminal lineage (Russell et al. 1990). Although organelle transmission appears to be the general case, Mogensen (1988) provided proof for the active exclusion of male germ cytoplasm in barley. He identified sperm

cytoplasm that had been shed within the degenerated synergid adjacent to the egg cell during nuclear transmission into the zygote. Interestingly, with respect to the central cell, sperm cytoplasm was not excluded, and thus within that barley cultivar, the endosperm receives sperm cells essentially intact, whereas the embryo apparently does not receive sperm cytoplasm.

Examination of plants reportedly having strictly maternal plastid inheritance reveals a typically small percentage of paternal organelle DNA being transmitted, despite overlapping mechanisms that favor the maternal inheritance of organelles in the embryo and endosperm. Prevalence of maternal organelles at a ratio that is between 1:100 and 1:1000 is not unusual, but makes the maintenance of biparental organelle transmission unlikely in most lineages, although a chimeral refugium of paternal genes may still be present despite these overwhelming numbers. The resistance of organelle DNA to be neatly contained even at fairly small numbers may allow escape of paternal organelles. There is also a concern of recombination, which occasionally occurs in organelles. A design defect in bicellular pollen may actually sequester cytoplasmic organelles within the nuclei of sperm cells (Yu and Russell 1993). In tobacco, generative cell division may not infrequently capture heritable organelles as inclusions in the sperm nuclei. That the pollen tube of tobacco is narrow enough to constrain the mitotic spindle during generative cell division may result in cytoplasmic organelles mingling with condensed chromosomes, and organelles may be incorporated into the reconstituted nucleus during nuclear reformation. Both plastids and mitochondria have been found in sperm nuclei (Yu and Russell 1994). There are also a few angiosperms that have strictly paternal inheritance of organelles, which arose by the placement of maternal organelles toward the basal lineage during embryogenesis, trapping female organelles in the terminal suspensor lineage, whereas male organelles are sequestered in the embryo proper (Mogensen 1996).

14.10 Chromatin Modeling: Expressional Control in Gametes, Embryo, and Endosperm

A common theme in the establishment of distinct cellular lineages in eukaryotes is chromatin modeling, where proteins that directly associate with DNA are altered in ways that influence gene expression. In *Arabidopsis*, CHR11 encodes an IMITATION OF SWITCH-like chromatin-remodeling protein that is abundantly expressed during female gametogenesis and later embryogenesis. Requirement of the activation of CHR11 was confirmed by producing an RNA interference construct to silence CHR11 transcripts. Under regulation by a functional megaspore promoter, the female gametophyte showed arrest of development (Huanca-Mamani et al. 2005). Heterochromatin abundance has long been described in the generative and sperm nuclei. This conversion involved chromatin remodeling encompassing the production of specialized histones, and particularly replication-independent substitution histones that displace normal histones. Such substitution histone

formation is a distinct marker of lineage differentiation in the male germ lineage, and is evident in both bicellular (lily, Okada et al. 2006a) and tricellular plants (*Arabidopsis*, Okada et al. 2006b). In these circumstances, histone H3 proteins are strategically expressed, and additionally are subjected to selective methylation as well. Lineage-specific chromatin remodeling through production of a substitution histone is also evident in the vegetative nucleus (Sano and Tanaka 2007).

The course of the reversal of such changes in the male lineage was directly visualized during and after fertilization by Ingouff et al. (2007), which revealed different modes of control in the zygote/embryo and endosperm. Using a male substitution histone H3.3::RFP protein construct, they monitored red fluorescence in fusion nuclei and their descendents to determine whether male substitution histone H3.3 was replaced or simply diluted following fertilization. Within the 7-h zygote, male histone H3.3 fluorescence was no longer detectable, suggesting active replacement by another replication-independent histone. In the endosperm, however, fluorescence decreased consistently with each round of DNA synthesis, suggesting dilution, rather than replacement of the male substitution histone. Thus, in the embryo lineage, replication-independent replacement histones appear to replace the fluorescent paternal histone H3.3, whereas the endosperm showed no evidence for histone replacement, except by normal replication-dependent replacement.

Chromatin modeling in the later endosperm nuclei suggests novel organization of chromatin, which may be related to its unique triploid construction with two copies of the maternal genome, and one copy of the paternal genome in *Arabidopsis*. By examining heterochromatin formation in endosperm nuclei, Baroux et al. (2007) found that there was a richness of additional heterochromatin centers and smaller chromocenters, perhaps reflecting a relaxed chromocenter-loop mode, and additional heterochromatic foci. They suggest that higher degrees of maternal heterochromatin production could reflect a means of differentially regulating maternal and paternal genes in a triploid, maternally rich environment.

14.11 Conclusions and Prospects

Double fertilization has occupied a crucial role in crop biology, particularly as most economically important plants use sexual means to reproduce. Considerable progress has been made in uncovering the molecules controlling sexual reproduction in angiosperms that account for most of our food crops. We now have information on genes involved with controlling pollen tube reception and discharge, cell cycle components relating to male germ cell control, and fertility controlling membrane proteins, providing a broadening knowledge of the biology of the male and female gametes and their products, the embryo and endosperm. Meeting our rising crop needs to extract greater productivity from shrinking supplies of arable land will depend on new knowledge. With this will also be a responsibility to provide the genetic variability required for assuring the sustainability of that success.

Within the next 5 years, the current rate of discovery suggests that information on the cellular and molecular control of sexual processes may hold key tools for biotechnology and crop improvement, and help us to understand how to better exploit these processes. As these tools of exploration continue to develop, in the very near future improved molecular knowledge of these topics relating to fertilization may allow unparalleled opportunities to manipulate crops to meet current and future human and environmental challenges.

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Chapter 15
Fruit Development

H. Ezura and K. Hiwasa-Tanase

15.1 Introduction

Fruit is a unique and important organ that developed in higher plants during evolution. Fruits protect seeds during development and serve as vehicles for seed dispersal to different habitats for species propagation (Tanksley 2004). Fleshy fruits are important in agricultural sciences because of their nutritional and economic values for humans. Important plant families that produce fleshy fruits include the Solanaceae (e.g. tomato, eggplant, pepper), the Cucurbitaceae (e.g. melon, cucumber, watermelon, pumpkin), the Roseaceae (e.g. apple, pear, strawberry), the Rutaceae (e.g. citrus) and the Vitaceae (e.g. grape).

Fleshy fruit development is essentially divided into four stages (Gillaspy et al. 1993), as depicted in Fig. 15.1. The first stage is floral development, which is the period from floral initiation to anthesis, when the identity, number and shape of floral organs are determined. The second stage is cell division, which commences with fertilization. Cell enlargement constitutes the third stage, when multiple rounds of endoreduplication and rapid cell expansion occur until the onset of ripening. Ripening is the fourth stage, which begins after fruit growth has ceased and involves rapid biochemical and structural changes that determine fruit aroma, texture, nutritional components (e.g. sugars, organic acids, amino acids) and colour.

During the last two decades, extensive molecular genetic and biochemical studies have revealed major mechanisms that underlie fruit development. In this chapter, fundamental aspects of each stage of fleshy fruit development and their biotechnological perspectives are discussed.

H. Ezura and K. Hiwasa-Tanase
Graduate School of Life and Environmental Sciences, University of Tsukuba, Tuskuba 305-8572, Japan
e-mail: ezura@gene.tsukuba.ac.jp

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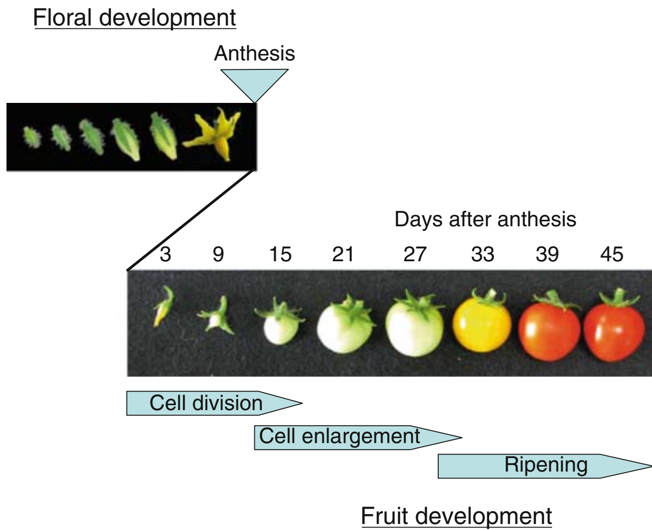

Fig. 15.1 Tomato floral and fruit development

15.2 Floral Development and Fruit Set

During floral development, the identity, number and shape of all floral organs are determined. Moreover, floral development has a profound effect on the final fruit size and shape. The genetic, developmental and molecular bases of fruit size and shape variation have been studied extensively in tomatoes, where key loci that control tomato size and shape, and genes relevant to fruit size and shape have been identified (Tanksley 2004).

15.2.1 Fruit Size

In tomato, fruit size is determined by the number of locules that develop from carpels after fertilization. Some tomato varieties produce more locules, resulting in a larger and wider fruit. Two loci, *fasciated* and *locule-number*, have been shown to control fruit size by determining the number of carpels in floral development (Tanksley 2004). Mutations at the *fasciated* locus appear to be more influential for fruit size than is the case for *locule-number* mutations. Most multilocular tomato varieties possess the *fasciated* mutation, but the production of extremely large fruit of some varieties requires the mutations of both *fasciated* and *locule-number*. Genetics studies have shown that the increase in carpel number associated with mutations of *fasciated* exhibits behaviour in a recessive manner, suggesting that such mutations render a loss-of-function mutant.

Near-isogenic lines have been used for a comparative developmental study of *fasciated* and wild-type tomato plants, with the aim to shed light on the nature of these changes. The *fasciated* gene was shown to affect floral meristem size directly, and was expressed before the earliest stages of flower organogenesis (Barrero et al. 2006). As a result, mature fruit of *fasciated* mutants showed more carpels (and locules), and greater fruit diameter and mass. Recently, a *fas* gene coding *fasciated* locus was isolated and characterized (Cong et al. 2008). The *fasciated* mutation was shown to result from a regulatory change in a YABBY-like transcription factor that controlled the carpel number during flower and/or fruit development. Furthermore, a multi-locular tomato variety transformed with the *FAS* gene showed a reduction in locules. These results suggest that fruit size can be genetically engineered, and knockdown of the *FAS* gene may lead to an increase in the fruit size of transgenic tomatoes.

15.2.2 Fruit Shape

Pear-shaped fruits are common in domesticated plants (Liu et al. 2002). A major quantitative trait locus (QTL) termed *ovate*, controlling the transition from round- to pear-shaped fruit, has been cloned from tomato. *OVATE* is expressed early during flower and fruit development. It encodes a previously uncharacterized, hydrophilic protein with a putative bipartite nuclear localization signal, Von Willebrand factor type C domains, and an approximately 70-amino acid C-terminal domain that is conserved in tomatoes, *Arabidopsis* and rice. A single mutation (GTA496-to-TTA493 polymorphism in the second exon) leading to a premature stop codon causes a round- to pear-shaped transition of tomato fruit shape (Liu et al. 2002). Moreover, ectopic expression of *OVATE* in transgenic plants reduced (variably) the size of floral organs and leaflets (Liu et al. 2002), suggesting that *OVATE* represents a previously uncharacterized class of negative regulatory proteins important in plant development. In addition, the gene *fs8.1* is responsible for the square tomato shape, and the slightly elongated appearance of processing tomatoes (Ku et al. 2000). Changes in the fruit shape conferred by this locus have been shown to begin early in floral/carpel development.

15.2.3 Fruit Set

Hormones play an important role in regulating fruit set after anthesis. The role of hormones in fruit setting is well described in tomato. For example, pollen produces gibberellins (GAs), and treatment of the tomato plant with GAs alone increases the auxin concentrations in the ovaries of unpollinated flowers, and triggers fruit set in the absence of fertilization (Gorguet et al. 2005). A crucial role for auxin in seedless tomato fruit formation was demonstrated by ovule-specific overexpression of an auxin biosynthetic gene, *iaaM*, from *Pseudomonas syringae* pv. *savastanoi*, leading to seedless fruit production independently of pollination (Rotino et al. 1997). *iaaM*

encodes the enzyme tryptophan mono-oxygenase that catalyses the conversion of tryptophan to indoleacetamide. Furthermore, tomato plants in which the Aux/IAA transcription factor *IAA9* was downregulated exhibited auxin-hypersensitivity (Wang et al. 2005). As a result, fruit was developed independently of pollination, leading to parthenocarpic fruits.

Parthenocarpy has been defined as the formation of seedless fruits in the absence of functional fertilization (Gorguet et al. 2005). It is an important trait for fruit crops. The production of seedless fruits is not only of great value for consumers for direct consumption, but is also important for the processing industry. Parthenocarpy is also advantageous when pollination or fertilization are deleteriously affected due to extreme temperatures. Unfortunately, mutations that cause parthenocarpic fruit development, as well as plant hormone-based approaches to obtain parthenocarpy often exhibit pleiotropic effects, and these plants usually possess undesirable characteristics such as misshapen fruits (Varoquaux et al. 2000; Fos et al. 2001; Wang et al. 2005). Recently, RNA interference (RNAi) was used to suppress the expression of the chalcone synthase gene, the first gene in the flavonoid biosynthetic pathway, leading to the development of parthenocarpic fruit (Schijlen et al. 2007). Hence, these results suggest that seedless plant varieties can be genetically engineered by downregulation of the key enzymes of the flavonoid biosynthetic pathway using RNAi-mediated approaches.

Several tomato mutant genotypes that result in parthenocarpic fruit growth have been described previously. The best described mutants and their genes include *pat* (Mazzucato et al. 2003), *pat2* (Philouze and Maisonneuve 1978), and *pat3* and *pat4* (Nuez et al. 1986). Parthenocarpic fruit development of all *pat* mutants occurs independently of pollination. In addition, some of these mutants (*pat*2, *pat*3 and *pat*4) contained high concentrations of GA in their fruits, suggesting a causal link between GA metabolism and parthenocarpic fruit development (Fos et al. 2000, 2001). Clearly, fruit development is controlled by complex processes, including the “cross talk” amongst multiple plant hormones.

15.3 Early Fruit Development

After fruit setting, cell division is activated in the ovary and continues until the end of the cell division phase. The cell division phase varies depending on the fruit species. The period of cell division influences the fruit cell number that, in turn, determines the final fruit size.

15.3.1 Cell Division and HMGRs

The relationships between cell division and fruit size have been well illustrated in melons. Cells of two different melon cultivars (*Cucumis melo* L. *reticulatus*) were studied during fruit development. Histological observations of the two genotypes,

Fuyu A and Natsu 4, revealed that the Fuyu A fruit was larger than that of Natsu 4 when grown under the same conditions, although their genetic backgrounds are nearly identical (Higashi et al. 1999). Microscopic observations of pericarp cells at several developmental stages revealed differences in cell size between the two genotypes. The cell proliferation period of Fuyu A was longer than that of Natsu 4, which may account for the difference in cell number between the genotypes. These results suggest that fruit size may be determined by the rate of cell proliferation at the early stage of fruit development. Genes related to cell division during the early stages of fruit development have been isolated. During the screening for ethylene receptor homologs in melon, a cDNA clone, designated *Cm-HMGR*, with high sequence homology to plant 3-hydroxy-3-methylglutaryl coenzyme A reductases (HMGRs), was serendipitously isolated (Kato-Emori et al. 2001). Functional expression of *Cm-HMGR* in HMGR-deficient mutant yeast demonstrated that the gene products mediated the synthesis of mevalonate. Northern analysis revealed that the level of *Cm-HMGR* mRNA in the fruit increased after pollination, and decreased sharply near the end of enlargement. During ripening, the level of *Cm-HMGR* mRNA increased markedly in the fruit. In parallel with mRNA accumulation, Cm-HMGR activity also increased after pollination at the early stage of fruit development, whereas Cm-HMGR activity was not detected during fruit ripening. These results suggest that *Cm-HMGR* may be important during early post-pollination fruit development in melons.

A detailed analysis of the correlation between the expression of the Cm-HMGR protein and cell division in the pericarp was also performed (Kobayashi et al. 2002). The findings strongly supported the hypothesis that Cm-HMGR expression is involved in determining the size of melon fruit by regulating cell division early in fruit development. In addition, the roles of *HMGR*s in fruit development have been examined using transgenic plants. Using *Agrobacterium*-mediated transformation, transgenic tomato plants that constitutively expressed a heterologous *HMGR* gene from melon *CmHMGR* were generated (Kobayashi et al. 2003). In the T_0 and T_1 generations of transgenic plants, the lines accumulated moderate levels of *CmHMGR* mRNA, and showed a significant increase in fruit size that resulted from a prolonged cell division period after pollination. In the T_4 transgenic line, plants with stable mRNA and protein of CmHMGR showed an increase in fruit fresh weight reaching 20% (Omura et al. 2007). A preliminary analysis of gene expression profile in transgenic and wild-type plants using cDNA macroarray was also conducted. The genes with the transcript level that showed more than threefold differences between transgenic and wild-type plants were identified. While 121 annotated genes and 152 genes of unknown function were differentially expressed, most genes involved in isoprenoid biosynthesis were not appreciably different.

15.3.2 Loci Associated with Cell Division

The two loci *fw2.2* and *sun* have been identified as QTLs that are associated with tomato fruit size and shape at the early stage of fruit development (Grandillo et al. 1999; van der Knaap and Tanksley 2001). *fw2.2* is a major QTL that can account for

up to 30% difference in fruit size between wild and cultivated tomatoes (Frary et al. 2000). Evidence thus far indicates that *fw2.2* alleles modulate fruit size through changes in gene regulation, rather than via specific actions of the FW2.2 protein itself. A detailed study was conducted using a pair of nearly isogenic lines of tomato to investigate the nature of these regulatory changes and the manner in which they may affect the fruit size (Cong et al. 2002). Results showed that the maximum level of transcript for the large- and small-fruited alleles of *fw2.2* differed chronologically by approximately 1 week. Moreover, this difference in timing of expression was associated with concomitant changes in mitotic activity at the early stage of fruit development. The differences in chronological gene expression (heterochronic allelic variation), combined with overall differences in total transcript levels, are sufficient to account for the majority of the phenotypic differences in fruit weight associated with the two alleles.

QTL mapping of chromosome 7 of one of the many processing tomato varieties revealed the presence of a second major fruit shape locus, which was named *sun* (van der Knaap and Tanksley 2001). Although allelic variation at both *ovate* and *sun* can cause the elongated fruit shape, the two loci differ in some important genetic, morphological and developmental aspects. First, *sun* causes uniform elongation in both longitudinal directions, such that the fruit maintains a bilateral symmetry, whereas *ovate* usually results in asymmetric elongation, such that the stem end of the fruit is more exaggerated than the blossom end. In addition, neck constriction or a pear shape has not been associated with this locus (van der Knaap et al. 2002). Second, developmental studies have shown that the changes in fruit shape associated with *sun* occurred after pollination and during the cell division stage of fruit development (van der Knaap and Tanksley 2001). In contrast, *ovate* acted much earlier during floral development. Alleles of *sun* interacted in an additive manner, raising the possibility that mutations that lead to elongated fruit are regulatory, rather than structural in nature (van der Knaap and Tanksley 2001).

15.4 Fruit Enlargement

15.4.1 Fruit Developmental Patterns

The fruit expands at non-uniform rates from setting to maturation. In most fruit, the developmental patterns are described as a single or double sigmoid curve (Fig. 15.2). The sigmoid curve developmental patterns occur when the increase in fresh weight or diameter of the fruit is plotted after anthesis. Fruits having single sigmoid curves undergo slow enlargement at the early and last stages of growth, while growth is considerably faster during the middle developmental stage. Examples of fruits with this developmental pattern include apples, pears, pineapples, bananas, avocados, strawberries, oranges, tomatoes and melons. On the other hand, fruits with the double sigmoid pattern exhibit two periods of rapid growth that are

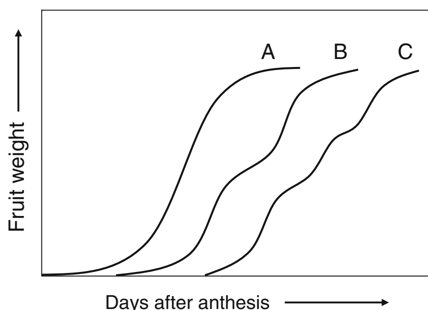

Fig. 15.2 Patterns of growth curve. *A* Single sigmoid growth curve, *B* double sigmoid growth curve, *C* triple sigmoid growth curve

separated by a period of relatively slow growth. This type of fruit includes all stone fruits, figs, blackcurrants, raspberries, blueberries, grapes and olives (Crane 1964; Coombe 1976). In addition, kiwifruit development showed a triple sigmoid growth curve (Coombe 1976; Fig. 15.2). Interestingly, the temporary plateau of growth in double sigmoid curve fruit types corresponds with seed hardening. This plateau, for the most part, does not occur in GA-induced parthenocarpic fruits, early season cultivars or hereditary seedless varieties. Therefore, seed development is thought to be related to the growth plateau.

15.4.2 Fruit Expansion

During the interval from anthesis to ripeness, fruits increase in volume and weight several thousand times. Notably, the avocado expands to a volume approximately 300,000 times the size of its ovary at anthesis (Kopp 1966; Coombe 1976). The size of fruit is determined fundamentally by the cell number and volume. The cell number depends on cell division, which becomes active from anthesis and fertilization. Cell division usually ends at the early stage of fruit maturity. Hence, early fruit development generally depends on cell division. Thereafter, growth is maintained by the expansion of each parenchyma cell. The magnitude of fruit cell enlargement has been reported to be influenced by water flow, cell wall plasticity and endoreduplication (Coombe 1976). In fruit parenchyma cells, vacuoles develop early after division and push the cytoplasm to the cell wall, where it adheres tightly. Some photosynthetic products synthesized in leaves during fruit development are stored in fruit parenchyma cells in the form of sucrose or sorbitol. Accumulation of sugar or sugar alcohols in parenchyma cells plays an important osmotic role in cellular water retention. When the cellular sugar concentration increases, this leads to an increase in the cell osmotic pressure and water flowing into vacuoles, thereby resulting in overall fruit expansion.

The cell wall is important for cell expansion. If the cell wall is too firm (not sufficiently pliant), the cell may have difficulty to take up water and, thus, cannot expand. For this reason, the cell wall must be flexible for cellular integrity and fruit

survival. Expansin is a candidate protein that promotes flexibility and plasticity in the cell wall (Cosgrove et al. 2002). This protein is localized in the cell wall and has been shown to promote plasticity of isolated plant walls in a pH-dependent manner in vitro, but it did not exhibit detectable hydrolase or transglycosylase activity (McQueen-Mason et al. 1992, 1993; McQueen-Mason and Cosgrove 1995). The biochemical and biophysical evidence suggests that expansins bind at the interface between cellulose microfibrils and matrix polysaccharides in the cell wall, disrupting hydrogen bonds within this polymeric network (McQueen-Mason and Cosgrove 1995). Nevertheless, the mechanism by which this protein acts in vivo is not clear. The mRNA and protein levels of the *expansin* gene were detected in growing tissues that elongated rapidly (Cho and Kende 1997a, b; Chen et al. 2001). Accumulation of *expansin* mRNA during fruit development has been reported in tomatoes, pears and grapes, suggesting the involvement of the expansin protein in fruit cell enlargement (Brummell et al. 1999; Catalá et al. 2000; Hiwasa et al. 2003b; Ishimaru et al. 2007).

Endoreduplication is a frequent somatic event in many plant tissues and organs (Kondorosi et al. 2000; Larkins et al. 2001; Barow and Meister 2003; Sugimoto-Shirasu and Roberts 2003). It has been known to occur in the fruit flesh cells of tomatoes, melons, apples and apricots (Bradley and Crane 1955; Kobayashi et al. 2002; Cheniclet et al. 2005; Harada et al. 2005). Endoreduplication is caused by chromosomal DNA replication without intervening mitoses. The degree of endoreduplication may affect cell growth, because the amount of cytoplasm is generally proportional to that of nuclear DNA (Sugimoto-Shirasu and Roberts 2003). Data obtained from 20 tomato lines showed that the DNA content in polyploid mature fruit cells can reach 512C (Cheniclet et al. 2005). Clearly, an increase in the ploidy level can influence cell and overall fruit size, as reported by Cheniclet et al. (2005), who showed a correlation between the mean cell size in the pericarp of various tomato genotypes and the mean ploidy level of developing fruit.

Recently, Gonzalez et al. (2007) reported a cell cycle-associated protein kinase, WEE1, which appears to play an important role as a regulator of tomato fruit development. The kinase activity at the interplay between normal cell cycle events and endoreduplication has been shown to influence the determination of organ and cell size. The role of WEE1 in fruit development has been demonstrated in transgenic tomato, where suppression of WEE1 expression caused a reduction in plant and fruit size, which might have resulted from a decrease in DNA ploidy levels and an overall reduction in cell size (Gonzalez et al. 2007). Moreover, WEE1 has been reported to regulate the timing of cell cycle events by inhibiting phosphorylation of cyclin-dependent kinases (CDKs; John and Qi 2008). In tomato fruits, large cells are found within the pericarp and the locular tissue that are characterized by arrested mitotic activity and inhibited CDK activity during the cell expansion phase of fruit development (Joubès et al. 1999, 2000, 2001).

Apples and figs have high volumetric capacity in their intercellular space. Therefore, in addition to cell number and expansion, the development of the intercellular space in these fruits is an important factor determining an overall

fruit size. In apples, the intercellular space increases proportionally and can make up to 27% of the fruit volume during fruit development (Bain and Robertson 1951).

15.4.3 Environmental Factors, Phytohormones and Fruit Growth

Division and expansion of fruit cells are determined genetically, at least in part. Nevertheless, appropriate environmental conditions (i.e. temperature, soil moisture content and cultivation management) during growth can allow fruits to grow to their maximum potential. Fruit growth is also influenced by hormones. Exogenous applications of auxin, which is known to control cell enlargement in plants, have been shown to alter the growth patterns and to increase the fruit size of apricots, blackberries, grapes, oranges, figs and prunes (Crane 1964). The seed inside of fruit is also capable of synthesizing auxin. In tomatoes (Gustafson 1939) and strawberries (Nitsch 1950), the auxin concentration in seeds is usually higher than that in other fruit tissues.

In addition to auxin, GA can accelerate fruit enlargement and maturation in parthenocarpic grapes, but the change in fruit enlargement rates is not universal in all fruit-bearing plants. For example, GA treatment of persimmon fruits at the end of the second phase or the beginning of the third phase of the growth curve can suppress fruit enlargement. In tomato, both auxin and GA are involved in fruit growth (Luckwill 1959). Hence, the effect of each hormone on the physiological state of the fruit is not uniform, and this depends on the fruit species, the time of application, and the relative content and balance of other plant hormones.

15.5 Fruit Maturation and Ripening

Most fruits modify their colour, texture, aroma and flavour during maturation. Fruits can be classified into two major groups, climacteric and non-climacteric, depending on whether or not there is increased respiration during maturation (Fig. 15.3). Climacteric fruits, such as apples, bananas, kiwis, pears, peaches, mangos, tomatoes and melons, exhibit an increase in respiration with a concomitant burst of ethylene production at the onset of ripening. In non-climacteric fruits such as citrus fruits, grapes and strawberries, on the other hand, increased respiration and ethylene production are observed during maturation.

15.5.1 Climacteric Fruit

An upsurge in respiration observed in apples at the end of the maturation phase was referred to as “climacteric” rise (Kidd and West 1930). Although “climacteric” was

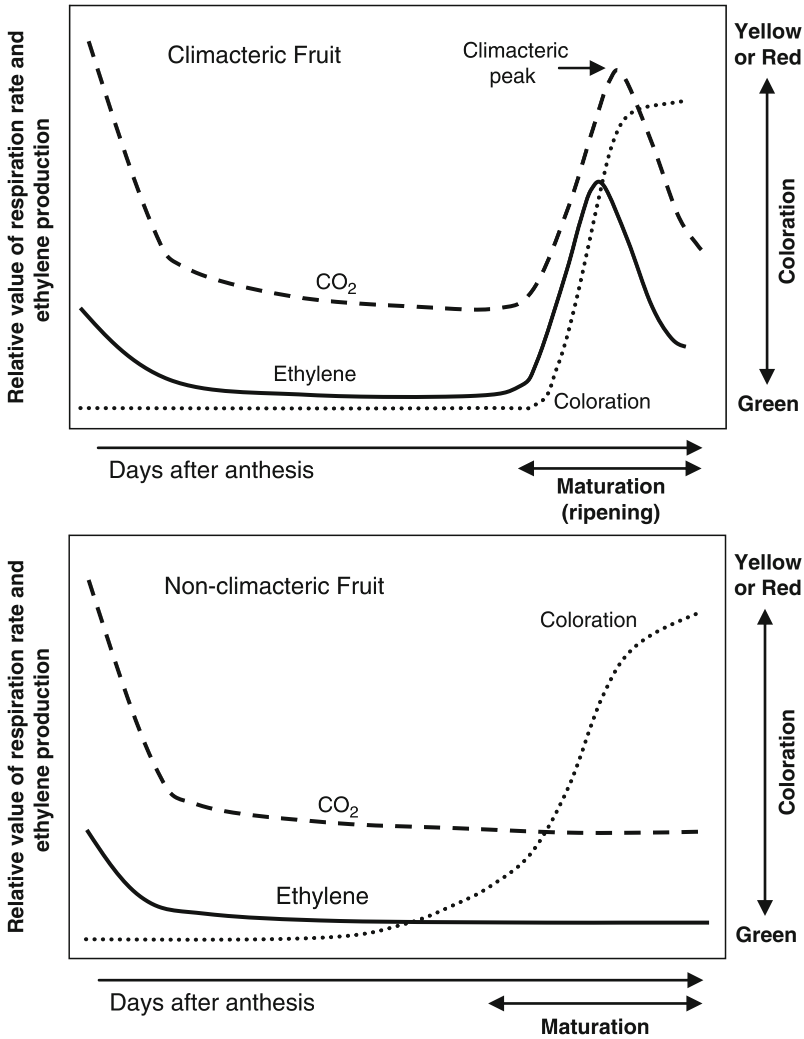

Fig. 15.3 Patterns of respiration rate, ethylene production, and coloration in climacteric and non-climacteric fruit

originally defined as an augmentation of respiration, it is now also characterized by a sudden increase in ethylene production at the onset of ripening. Ethylene is a gaseous plant hormone that plays an important regulatory role in various plant physiological processes, including fruit ripening (Yang and Hoffman 1984). In climacteric fruits, increased ethylene production usually coincides with changes in colour, aroma, texture, flavour, and other biochemical and physiological fruit attributes. The rapidity by which ethylene promotes ripening was demonstrated in the Charentais variety of melon (*Cucumis melo* cv. *reticulatus* F1 Alpha), where the

ripening process from the pre-ripe to overripe stage occurred within 24–48 h (Rose et al. 1998). Exposure of climacteric fruit, even at the pre-climacteric stage, to exogenous ethylene can induce a rapid increase in autocatalytic ethylene production, thereby accelerating the ripening process. In contrast, treatment of fruits at the pre-ripe or ripe stage with ethylene action inhibitors can suppress ethylene-induced responses, decrease respiration and delay maturation (Blankenship and Dole 2003). Results of these studies confirmed the pivotal role of ethylene in regulating ripening of climacteric fruit.

An understanding of the regulatory networking in biosynthesis and signal transduction of ethylene is important for the elucidation of mechanisms that regulate fruit ripening. The process of fruit ripening has been studied intensively using ripening mutants, transgenic analyses, and other genetic and molecular tools (Leliévre et al. 1997; Johnson and Ecker 1998; Giovannoni 2001, 2004, 2007; Klee 2002; Alexander and Grierson 2002). In Charentais melon, ethylene production was inhibited by downregulation of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase expression. Modulation of ethylene production was shown to affect fruit ripening, in terms of yellowing of the rind, softening of the flesh, development of the peduncular abscission zone, aroma formation and climacteric respiration (Ayub et al. 1996; Guis et al. 1997; Bauchot et al. 1998; Bower et al. 2002; Pech et al. 2008). However, pulp coloration, accumulation of sugars, and loss of acidity appeared to be ethylene-independent, indicating that climacteric fruit ripening involved both ethylene-dependent and ethylene-independent processes.

15.5.2 Non-Climacteric Fruit

In non-climacteric fruits, ripening progresses gradually, which renders long-term storage of these fruits possible. Ethylene is generally not required for fruit maturation and ripening. Nevertheless, exposure of fruits to exogenous ethylene has been shown to stimulate chlorophyll degradation (Purvis and Barmore 1981) and carotenoid biosynthesis (Stewart and Wheaton 1972). Furthermore, treatments with the ethylene antagonists 2,5-norbornadiene and silver nitrate prevented the de-greening process (Goldschmidt et al. 1993), underscoring a role for ethylene in some non-climacteric fruits. Biale (1964) reported an increase in the respiration rates of non-climacteric fruits in response to ethylene, but this ethylene-stimulated respiration ceased upon removal of ethylene (Leliévre et al. 1997). In general, non-climacteric fruits do not produce substantial amounts of ethylene at the maturing stage. Nonetheless, they have the capacity to produce some endogenous ethylene and to respond to exogenous ethylene.

The mechanism that regulates the maturation and ripening in non-climacteric fruit is not clear, but it may be associated with changes in the concentrations of auxin, GA and abscisic acid (ABA). In grape, the “veraison” stage occurs when the berry size and concentrations of anthocyanin and sugars increase markedly during

fruit growth and maturation. The ripening of grape could be induced by treatment with ABA but suppressed by auxin (Inaba et al. 1976), indicating that ABA and auxin may be involved in the regulation of grape ripening.

15.5.3 Changes in Fruit Composition

15.5.3.1 Sugars and Organic Acids

During fruit maturation, sugar concentrations reach a peak, whereas the organic acid content decreases. Two types of sugar accumulation can be recognized: one is gradual sugar accumulation until the fruits reach the maturity stage (e.g. mandarin oranges, apples and tomatoes), and the other type is rapid sugar accumulation at the later developmental stage of the fruit (e.g. grapes, peaches and melons). Although all ripe fruits generally contain relatively high concentrations of sugars, the sugar composition differs. Peaches and melons accumulate mainly sucrose, while tomatoes, grapes and apples accumulate predominantly reducing sugars, such as glucose and fructose. Moreover, most fruits also contain organic acids, such as citric acid and malic acid. However, grapes usually accumulate tartaric acid, rather than citric acid. The concentration of organic acid in ripening fruit is usually 1% or less, with the exception of lemons.

15.5.3.2 Amino Acids and Volatiles

During fruit maturation, free amino acids increase and contribute to the formation of a peculiar taste. Tomatoes contain umami components, such as glutamic and aspartic acids, as the major amino acids. Apples, peaches, pears and apricots contain large amounts of aspartic acid or asparagine, while grapes and citrus fruits are rich in proline and arginine. The volatile components of fruit that contribute to flavour also increase during fruit maturation. Fruit aromas are determined mainly by the composition of volatiles such as alcohols, esters, carbonyls and terpenes.

15.5.3.3 Pigments and Fruit Softening

The main pigments present in mature fruits are chlorophyll (in kiwis and avocados), anthocyanins (in apples, plums, blueberries and grapes), carotenoids (in citrus fruits, bananas, persimmons and tomatoes) and flavonoids (in citrus fruits). Chlorophyll content decreases during fruit development and maturation. The yellow colour of bananas is the result of carotenoids that accumulate before ripening, and chlorophyll degradation that occurs during maturation. Similarly, the colour of

citrus fruits is caused by chlorophyll degradation and an increase in carotenoid synthesis during maturation.

Decrease in fruit firmness is typical during maturation and ripening. The softening process proceeds quickly in climacteric fruits (e.g. pears and melons) at the onset of ripening (Rose et al. 1998; Hiwasa et al. 2003a). Fruit softening is caused by the depolymerization and solubilization of cell wall polysaccharides, which are classified broadly into pectins, hemicelluloses and cellulose. During fruit softening, pectins (Fischer and Bennett 1991) and hemicelluloses (Wakabayashi 2000) undergo solubilization and depolymerization, whereas changes in cellulose are negligible.

15.6 Perspectives

Comprehensive studies on fruit development using model plants are ongoing. Specifically, tomato plants are excellent models for fruit development. In 2003, the International Solanaceae Project (SOL; http://www.sgn.cornell.edu) was initiated by members from more than 30 countries, and the tomato genome-sequencing project is currently under way. The fully sequenced tomato genome will provide a firm foundation for forthcoming genomic studies dealing with, for example, the comparative analysis of genes conserved amongst the Solanaceae, and the elucidation of the functions of unknown tomato genes. To exploit the wealth of genome sequence information, an urgent need exists for novel resources and analytical tools to use in tomato functional genomics, including genetic linkage maps, DNA markers, expressed sequence tags, full-length cDNA, gene expression data, mutant resource, and the TILLING platform, a high-efficiency transformation system (Sun et al. 2006) that was summarized by Matsukura et al. (2008).

Melons are an alternative model for elucidating the regulatory mechanism of fruit development. Several tools are available for melon functional genomics, such as the bacterial artificial chromosome (BAC) library (Luo et al. 2001; van Leeuwen et al. 2003), expressed sequence tag (EST) collections (Gonzalez-Ibeas et al. 2007) and EcoTILLING (Nieto et al. 2007). In addition, the International Cucurbit Genomics Initiative (ICuGI) was launched in 2005 using melons as model plants. The objective of this initiative is to build a platform for cucurbit genomics, functional genomics (sequencing of 100,000 ESTs from different melon genotypes and tissues), mapping (merging the existing melon genetic maps using simple sequence repeat motifs, SSRs, as anchor markers) and bioinformatics (development of a webpage for the ICuGI, where certain genomic tools would be available for the cucurbit research community; http://www.icugi.org/). Furthermore, development of other tools are in progress for melon functional genomics, including ethylmethanesulfonate (EMS) mutant families, DNA pools for TILLING (Japan

and other countries) and the optimized melon transformation method using *Agrobacterium* (Nonaka et al. 2008a, b). In general, these tools and the knowledge gained from studies of model plants may help to elucidate the molecular mechanisms of fruit development.

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Chapter 16
Mechanism of Fruit Ripening

M. Bouzayen, A. Latché, P. Nath, and J.C. Pech

16.1 Introduction: Fruit Ripening as a Developmentally Regulated Process

The making of a fruit is a developmental process unique to plants. It requires a complex network of interacting genes and signaling pathways. In fleshy fruit, it involves three distinct stages, namely, fruit set, fruit development, and fruit ripening. Of these, ripening has received most attention from geneticists and breeders, as this important process activates a whole set of biochemical pathways that make the fruit attractive, desirable, and edible for consumers. In recent years, the scientific goal has been to reveal the mechanisms by which nutritional and sensory qualities are developed during fruit development and ripening using advanced genomics and post-genomics tools. These genome-wide technologies have been combined to physiological approaches to decipher the networks of interactions between the different pathways leading to the buildup of fruit quality traits. From a scientific point of view, fruit ripening is seen as a process in which the biochemistry and physiology of the organ are developmentally altered to influence appearance, texture, flavor, and aroma (Giovanonni 2001, 2004). For the consumers and distributors, the process of ripening corresponds to those modifications that allow fruit to become edible and attractive for consumption. Since the majority of the quality attributes are elaborated during the ripening process, it has always been considered

M. Bouzayen
Génomique et Biotechnologie des Fruits, INRA, Chemin de Borde Rouge, F-31326, Castanet-Tolosan, France
e-mail: bouzayen@ensat.fr

A. Latché and J.C. Pech
Génomique et Biotechnologie des Fruits, Université de Toulouse, INP-ENSA Toulouse, Avenue de l'Agrobiopole, BP 32607, F-31326 Castanet-Tolosan, France

P. Nath
Plant Gene Expression Laboratory, National Botanical Research Institute, Rana Pratap Marg, Lucknow 226 001, India

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essential to better understand the mechanisms underlying this ultimate fruit developmental stage.

The fruit ripening process has been viewed over the last decades as being successively of physiological, biochemical, and molecular nature. Fruit ripening is accompanied by a number of biochemical events, including changes in color, sugar, acidity, texture, and aroma volatiles that are crucial for the sensory quality (Fig. 16.1). At the late stages of ripening, some senescence-related physiological changes occur that lead to membrane deterioration and cell death. In that regard, fruit ripening can thus be considered as the first step of a programmed cell death process. All biochemical and physiological changes that take place during fruit ripening are driven by the coordinated expression of fruit ripening-related genes. These genes encode enzymes that participate directly in biochemical and physiological changes. They also encode regulatory proteins that participate in the signaling pathways, and in the transcriptional machinery that regulate gene expression and set in motion the ripening developmental program (Fig. 16.1).

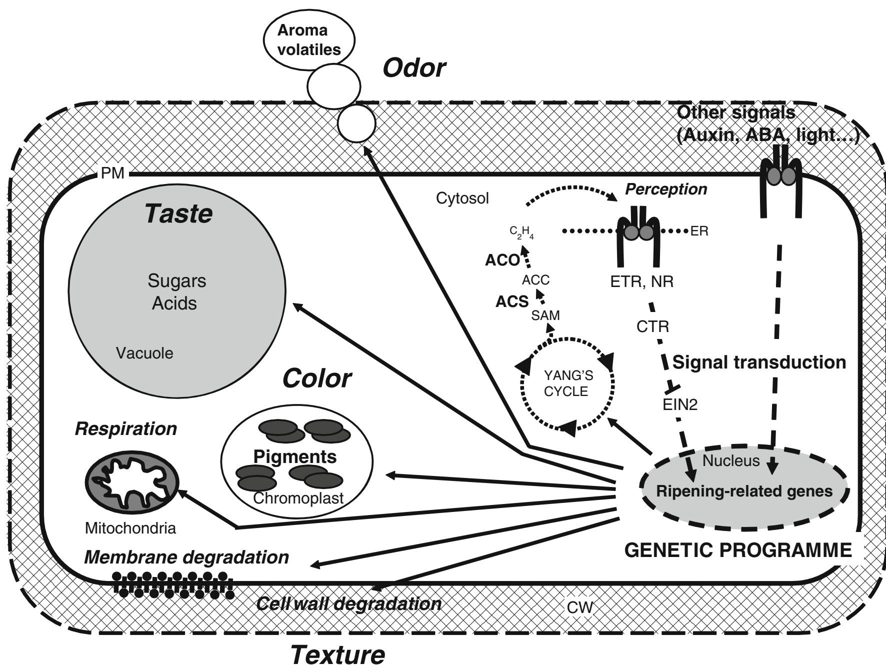

Fig. 16.1 Schematic representation depicting the molecular mechanisms controlling the ripening of climacteric fruit. The fruit ripening process is a genetically regulated developmental process involving the activation of a high number of primary and secondary metabolic pathways that all contribute to the overall sensory and nutritional quality of the fruit. This process involves the expression of ripening-related genes that encode enzymes (proteins) involved in the various ripening pathways (e.g., softening, color development). The whole process is under the control of hormonal and environmental signals, amongst which ethylene plays a major role

16.2 Climacteric and Non-Climacteric Fruit Ripening

Fruit can be divided into two groups according to the regulatory mechanisms underlying the ripening process. Climacteric fruit, such as tomato, apple, pear, and melon (Table 16.1), are characterized by a ripening-associated increase in respiration and in ethylene production. By contrast, non-climacteric fruits, such as orange, grape, and pineapple (Table 16.1), are characterized by the lack of ethylene-associated respiratory peak. At the onset of ripening, climacteric fruit present a peak in respiration, and a concomitant burst of ethylene production. The relationship existing between the climacteric respiration and fruit ripening has been questioned following the discovery that ripening on the vine of a number of fruit may occur in the absence of any increase in respiration (Salveit 1993; Shellie and Salveit 1993). More recently, it has been reported that the presence or absence of a respiratory climacteric on the vine depends upon prevailing environmental conditions (Bower et al. 2002). These observations indicate that the respiratory climacteric is probably not an absolute trigger of the ripening process, but secondary and consequential to the process of ripening. An ethylene burst that precedes respiratory climacteric has been shown during the ripening of banana (Pathak et al. 2003).

Table 16.1 A list of representative climacteric and non-climacteric fruit. A more extensive list is provided by Watkins (2002)

Climacteric fruits	Non-climacteric fruits
Apple (*Malus domestica* Borkh.)	Asian pear (*Pyrus serotina* Rehder)
Apricot (*Prunus armeniaca* L.)	Cactus pear (*Opuntia amyclaea* Tenore)
Avocado (*Persea americana* Mill.)	Carambola (*Averrhoa carambola* L.)
Banana (*Musa sapientum* L.)	Cashew (*Anacardium occidentale* L.)
Cherimoya (*Annona cherimola* Mill.)	Cherry (*Prunus avium* L.)
Corossol (*Annona muricata* L.)	Cucumber (*Cucumis sativus* L.)
Durian (*Durio zibethinus* Murr.)	Grape (*Vitis vinifera* L.)
Feijoa (*Feijoa sellowiana* Berg.)	Grapefruit (*Citrus grandis* Osbech)
Fig (*Ficus carica* L.)	Lime (*Citrus aurantifolia* Swingle)
Guava (*Psidium guajava* L.)	Limon (*Citrus limonia* Burm.)
Kiwifruit (*Actinidia sinensis* Planch.)	Litchee (*Litchi sinensis* Sonn.)
Mango (*Mangifera indica* L.)	Mandarin (*Citrus reticulata* Blanco)
Melon Cantaloup and Honeydew (*Cucumis melo* L.)	Mangoustan (*Garcinia mangostana* L.)
Papaya (*Carica papaya* L.)	Olive (*Olea europaea* L.)
Passion fruit (*Passiflora edulis* Sims.)	Orange (*Citrus sinensis* Osbeck)
Peach (*Prunus persica* Batsch)	Pepper (*Capsicum annuum* L.)
Pear (*Pyrus communis* L.)	Pineapple (*Ananas comosus* Merr.)
Persimmon (*Diospyros kaki* Thunb.)	Pomegranate (*Punica granatum* L.)
Physalis (*Physalis peruviana* L.)	Rambutan (*Nephelium lappaceum* L.)
Plum (*Prunus domestica* L.)	Raspberry (*Rubus idaeus* L.)
Sapota (*Manilkara achras* Fosb.)	Strawberry (*Fragaria* sp.)
Tomato (*Solanum lycopersicum L.*)	Tamarillo (*Cyphomandra betacea* Sendtu)
	Watermelon (*Citrullus lanatus* Mansf.)

16.2.1 Ethylene Production, and Its Role in Climacteric and Non-Climacteric Fruit

Two distinct ethylene biosynthesis systems have been described. System 1 corresponds to low ethylene production in the pre-climacteric period of climacteric fruit, and is present throughout the development of non-climacteric fruit. System 2 refers to an auto-stimulated massive ethylene production called "autocatalytic synthesis", and is specific to climacteric fruit. Therefore, the major ethylene-related differences between climacteric and non-climacteric fruit is the presence or absence of autocatalytic ethylene production (McMurchie et al. 1972; Alexander and Grierson 2002). The ethylene biosynthetic pathway is now well established (Fig. 16.1; Yang and Hoffmann 1984). This ripening hormone is synthesized from methionine via *S*-adenosyl-L-methionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC). Two major enzymes are involved in the biosynthetic pathway, namely, ACC synthase (ACS), which converts SAM into ACC, and ACC oxidase (ACO), which converts ACC into ethylene. The corresponding genes have been identified and characterized (Sato and Theologis 1989; Hamilton et al. 1990, 1991). Both ACO and ACS are encoded by a multigene family of five and nine members, respectively in tomato, with expressions differentially regulated during fruit development and ripening (Barry et al. 1996, 2000). While *LeACO1* and *LeACO4* genes are up-regulated at the onset of ripening, and continue being active throughout ripening, *LeACO3* displays only transient activation at the breaker stage of fruit ripening (Fig. 16.2). It was shown that *Le ACS6* and *LeACS1A* are expressed at the pre-climacteric stage (system 1), while at the transition to ripening, *LeACS4* and *LeACS1A* are the most active genes (Fig. 16.2). Subsequently, *LeACS4* continues to express highly during climacteric phase, whereas the expression of *LeACS1A* declines. The rise in ripening-associated ethylene production results in the induction of *LeACS2*, and the inhibition of *Le ACS6* and *LeACS1A* expression. This fine tuning of the *ACS* genes is thought to be critical for the switch from pre-climacteric system 1 to climacteric system 2. Noteworthy is that system 1 is characterized by inhibitory feedback of ethylene in its own biosynthetic pathway, whereas the transition to system 2 is characterized by autocatalytic production. The requirement for ethylene to trigger the ripening of climacteric fruit has been clearly demonstrated by down-regulating *ACO* and *ACS* genes in transgenic plants using an antisense strategy. The ethylene-suppressed lines showed strongly delayed ripening in tomato (Oeller et al. 1991; Picton et al. 1993), and in other fruits, e.g., melon (Ayub et al. 1996) and apple (Dandekar et al. 2004). However, ethylene-independent ripening pathways exist in climacteric fruit, as illustrated in melon fruit, where part of softening, sugar accumulation, and coloration of the flesh occur in ethylene-suppressed fruit (Flores et al. 2001). These results have led to the conclusion that climacteric (ethylene-dependent) and non-climacteric (ethylene-independent) regulation coexists in climacteric fruit (Pech et al. 2008a).

Although the ripening of non-climacteric fruit is not associated with any significant change in ethylene production, some ethylene-dependent processes do exist in

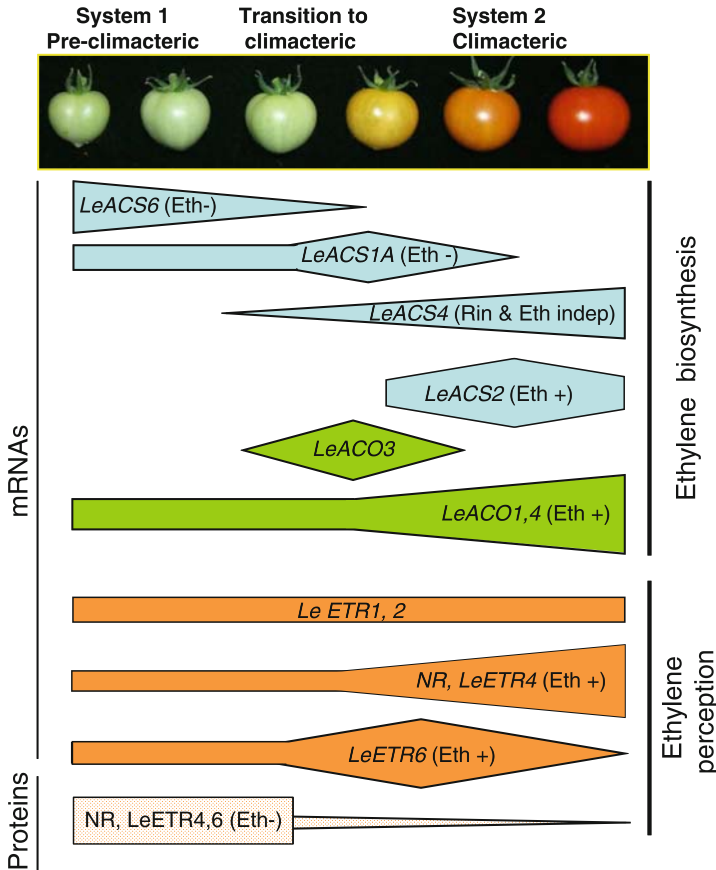

Fig. 16.2 Schematic representation describing the expression of ethylene biosynthesis and ethylene perception genes during the transition to climacteric in tomato. System 1 refers to pre-climacteric ethylene production, and System 2 to climacteric autocatalytic ethylene production. LeACS, *Lycopersicon esculentum* ACC synthase; LeACO, *Lycopersicon esculentum* ACC oxidase; LeETR and NR, ethylene receptors. Eth+ and Eth– refer to the stimulation and repression, respectively of gene or protein expression (adapted from Barry et al. 1996, 2000; Kevany et al. 2007)

this type of fruit. In grape berries, the ethylene synthesis pathway is activated at the inception of the ripening, the so-called veraison stage. Treatments with exogenous ethylene stimulate the long-term expression of genes related to anthocyanin synthesis, and ethylene signals appear to be involved in the regulation of vascular flux, acid content, and in some steps of aroma volatile production (Mailhac and Chervin 2006). In citrus, another class of typically non-climacteric fruit, the existence of an autocatalytic system of ethylene production similar to that of climacteric fruit has been suggested (Katz et al. 2004), and it is well known that these types of fruit have the ability to respond to exogenous ethylene in terms of chlorophyll degradation. Moreover, in all non-climacteric fruits, exogenous ethylene accelerates senescence

via the deterioration of cell membranes. While the role of ethylene in climacteric fruit ripening is beginning to be well understood, the main signaling pathways involved in non-climacteric ripening remain very poorly understood.

16.2.2 Ethylene Perception and Signal Transduction

Breakthrough advances in the field of ethylene perception have been made possible by the use of the model plant Arabidopsis, and the implementation of molecular genetics strategies. Following the identification of the ethylene-insensitive mutants, named ETR1 (Bleecker et al. 1988), the gene encoding the ethylene receptor was isolated by positional cloning (Chang et al. 1993). The ethylene receptor was the first plant hormone receptor to be isolated and characterized, and this paved the way toward the isolation of the other components of the ethylene transduction pathway (Klee and Clark 2004). Based on these discoveries, the use of Arabidopsis has been critical in helping to isolate the ethylene receptor from other plant species, and to understand the role of the receptors in the ripening process. The ethylene receptors are encoded by a small multigene family for structurally distinct but functionally redundant proteins working either as hetero- or homo-multimers. In tomato, six ethylene receptor genes have been isolated and found to be expressed in all plant tissues, three of these showing a net increase during ripening, while two express constitutively (Fig. 16.2). Interestingly, it was demonstrated that the tomato *Never ripe* (*Nr*) mutation, which results in impaired ripening, occurs in one of the ethylene receptor genes. Recent studies demonstrated that the ethylene receptors are rapidly degraded during fruit ripening, while the transcription rate remains high, and that the receptor level determines the timing of ripening (Kevany et al. 2007). Moreover, the suppression of the ethylene receptor LeETR4 led to an early ripening of tomato fruits (Kevany et al. 2008).

In more applied terms, the search for ethylene antagonists led to the discovery of 1-methylcyclopropene (MCP), a powerful antagonist of ethylene action (Sisler et al. 1999). This compound is now widely used both by academic researchers as a tool for understanding ethylene-regulated developmental processes (Blankenship and Dole 2003), and by the producers and shippers of fresh fruit and flowers on a commercial scale for extending the shelf life of these products. MCP probably represents the most remarkable innovation in the past two decades in the field of post-harvest horticulture (http://www.hort.cornell.edu/department/faculty/watkins/ethylene/).

The *CTR1* gene (*Constitutive Triple Response*), first isolated from Arabidopsis, encodes another major component of ethylene signaling lying downstream of the receptor acting as a negative regulator of the ethylene transduction pathway (Kieber et al. 1993). The tomato *CTR1* gene (*Sl-CTR1*) was first isolated from fruit tissue (Leclercq et al. 2002), and in spite of being a negative regulator of ethylene responses, its transcripts are up-regulated during fruit ripening, commensurate with the rise in ethylene production. Subsequently, it was shown that the CTR

family was composed of four genes in tomato, each displaying a specific pattern of expression during ripening and in response to ethylene, with *Sl-CTR1* being the most actively expressed during fruit ripening (Adams-Phillips et al. 2004). Strikingly, reverse genetic strategies have to date failed to show any impact of altered *CTR1* expression on the fruit ripening process, indicating a potential functional redundancy among the *CTR* genes.

16.2.3 Control of Ethylene Response in Fruit

Because of the tremendous change in the expression level of a large number of genes during fruit ripening, and in order to gain better insight into the control mechanisms underlying this process, differential screening approaches were attempted to isolate and characterize ethylene-regulated genes (Lincoln et al. 1987). Genes encoding cell wall-degrading, ethylene production, and pigment biosynthesis enzymes were among the first ethylene-responsive genes to be isolated from tomato fruit. Later, a set of early ethylene-regulated genes were isolated from mature green tomatoes that are responsive to exogenous ethylene, but not yet producing elevated levels of ripening-associated ethylene (Zegzouti et al. 1999). Expression studies revealed that the ethylene-responsive genes can be up-regulated, down-regulated, or transiently induced following short periods of hormone treatment, supporting the idea that ethylene can act as negative or positive regulator of gene expression (Gupta et al. 2006; Kesari et al. 2007). Noteworthy is that many of the early ethylene-responsive genes encode putative regulatory proteins involved in transduction pathways and transcriptional or post-transcriptional regulation, indicating that the ethylene control of the ripening process operates in a complex multilevel way. More recently, the work by Giovannoni's group (Alba et al. 2005) demonstrated the importance of ethylene control during tomato fruit development. In the tomato *Nr* mutant, impaired in ethylene sensing and fruit ripening, up to one third of ripening-associated genes showed altered expression compared to wild type (Alba et al. 2005). Moreover, in a non-climacteric fruit like strawberry, microarray analyses comparing akene and receptacle tissues show high levels of ethylene response factor (*ERF*) and ethylene regulated (*ER*) gene expression in akene tissue, suggesting a role for ethylene in the maturation of the akene (Aharoni and O'Connell 2002). Together, these data demonstrate the important role of ethylene in fruit ripening in both climacteric and non climacteric fruit. However, the mechanistic insight into how ethylene acts to bring about the activation of all the ripening-associated metabolic pathways remains unclear. Ethylene is known to have numerous effects on a wide range of developmental processes, including germination, flower and leaf senescence, fruit ripening, leaf abscission, root nodulation, programmed cell death, and responsiveness to abiotic stress and pathogen attack (Johnson and Ecker 1998; Bleecker and Kende 2000; Pirrello et al. 2006). This diversity of plant responses to ethylene raises the question on how this phytohormone selects the desired target genes with respect to their tissue and

developmental specificity. This question becomes even more relevant when considering that the ethylene transduction pathway is linear in its upstream part from the receptor to *ein3*, the first transcription regulator. It is therefore tempting to speculate that most of the diversity of ethylene responses may arise largely from fine tuning of the expression and/or activity of ERFs, transcriptional regulator proteins lying downstream of EIN3 (Fig. 16.3). Indeed, ERFs belong to one of the largest families of transcription factors in plants (Riechmann et al. 2000), thus offering different branching possibilities to channel the hormone signaling to a variety of responses. The diversity and complexity of ethylene responses can also arise from the cross-talk between ethylene and other hormones (Rosado et al. 2006; Stepanova et al. 2007).

ERF genes encode a type of *trans*-acting factors unique to plants that specifically bind the GCC box, a conserved motif of the *cis*-acting element found in the promoter of ethylene-responsive genes (Ohme-Takagi and Shinshi 1995; Solano et al. 1998). ERFs are known to be the last actors of the ethylene signaling pathway,

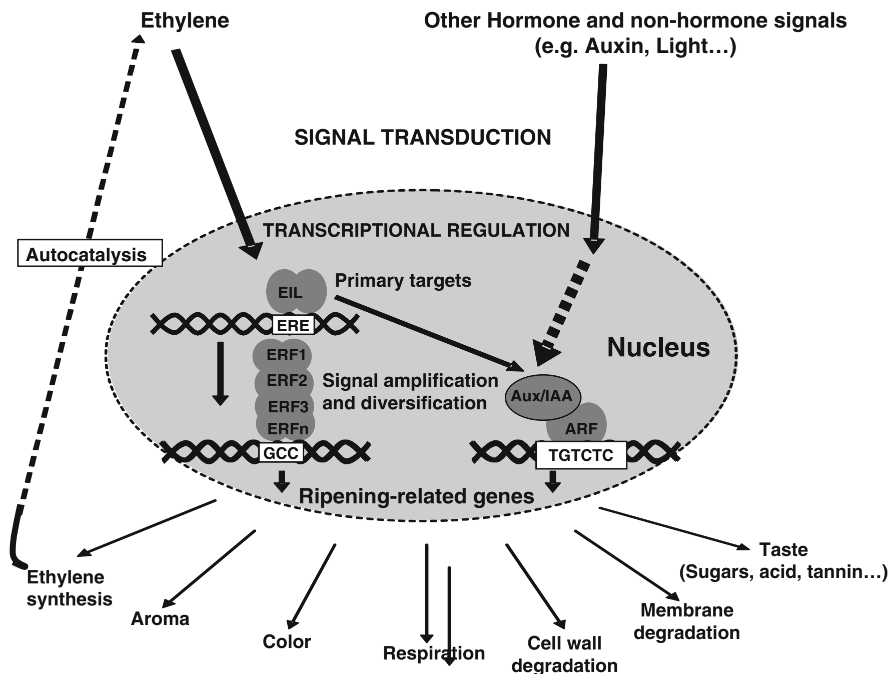

Fig. 16.3 The hormone-dependent transcriptional regulation associated with fruit ripening. A main focus is made on ethylene and auxin, aiming at exemplifying the importance of cross-talk between hormone signaling. Ethylene transduction cascade leads to the activation of *EIN3-Like* (*EIL*) genes, which activates primary target genes (ethylene-response factors, ERFs). ERFs in turn activate the expression of secondary ripening-related genes. Other signals, such as auxin, are also involved in this process. Some auxin response factors (ARFs) and Aux/IAA transcription factors are also ethylene-responsive, and therefore are likely to participate in the expression of ripening-related genes (Jones et al. 2002)

and the ERF family is part of the AP2/ERF superfamily of transcription factors, which also contains the AP2 and RAV families (Riechmann et al. 2000). Since *ERFs* belong to a large multigene family, it is expected that members of this family have varied functionality, and diverse binding activities. Using combined reverse genetics and transcriptomics approaches, intensive studies are in progress to uncover the specific role of each ERF in the ripening process, and to establish the set of target genes regulated by each member of this transcription factor family. In the long term, the objective of these studies is to set up tools enabling targeted control of the ripening process, thus allowing engineering fruit ripening in specific ways, such as slowing down the loss of firmness, while enhancing desired metabolic pathways.

16.3 Hormone Cross-Talk and Fruit Ripening

As mentioned above, fruit ontogeny and ripening are genetically regulated processes involving a complex multi-hormonal control (Fig. 16.3). While the roles of ethylene in triggering and regulating the ripening of climacteric fruit have been clearly demonstrated, little is known on the roles of other hormones. Phytohormones exert their effect on plant development via a chain of transduction pathways that ultimately activates specific transcription factors, which in turn regulate the expression of a set of target genes. In order to uncover the role of hormones that act in concert with ethylene to regulate tomato fruit development, a screen for transcription factors showing differential expression from fruit set through ripening led to the isolation of a number of genes encoding auxin transcriptional regulators of the ARF and Aux/IAA type (Jones et al. 2002). Among the isolated auxin-response factors, some showed fruit-specific and ethylene-regulated expression that clearly correlated with their pattern of ethylene responsiveness, suggesting a cross-talk between ethylene and auxin throughout fruit development (Jones et al. 2002; Wang et al. 2005). Combined reverse genetic and transcriptomic approaches have been carried out to uncover the functional significance of these genes. Molecular and physiological characterization of transgenic tomato plants under- and over-expressing these transcription factors confirmed their crucial role in both early and late stages of fruit development. Important quality traits, such as sugar content, firmness, and parthenocarpy, are strongly affected in the transgenic lines (Jones et al. 2002; Wang et al. 2005). These genes offer new potential targets for improving fruit quality, either by marker-assisted selection or by biotechnological means.

16.4 Biochemical Changes and Sensory Traits Associated with Fruit Ripening

One of the major factors associated with the post-harvest deterioration of fruit is the rate of softening. Excessive softening results in shorter shelf life during storage, transportation and distribution, and increased wastage. A number of genes potentially

involved in cell wall degradation, rearrangement and structure have been isolated, and most of these have been studied in the tomato model. However, unexpectedly, it has been shown that the suppression of candidate genes, such as those encoding polygalacturonase, pectin-methyl-esterase, and β-glucanase, did not have a major impact on the evolution of fruit firmness (Giovannoni et al. 1989; Tieman et al. 1992; Brummell et al. 1999a). Up to 40% reduction of tomato fruit softening has been achieved by down-regulating the *TBG4* β-galactosidase gene (Smith et al. 2002), but in antisense *TBG4* fruit, *TBG3* gene expression was also reduced, indicating a possible cooperation of the two genes. Expansins are cell wall proteins that loosen cell walls by reversibly disrupting hydrogen bonds between cellulose microfibrils and matrix polysaccharides. The *LeExp1* (*tomato expansin 1*) gene encodes a protein that is specifically expressed in ripening fruit. Down-regulation resulted in strong reduction of softening throughout ripening, probably by alteration of the microfibril-/matrix glycan interface that facilitates access of cell wall hydrolases to the matrix glycan substrates (Brummell et al. 1999b). Another class of cell wall-degrading enzymes, pectate lyases, appears to have a more important role in ripening than previously expected. In strawberry, a non-climacteric fruit, suppression of the pectate lyase mRNA resulted in significantly firmer fruits (Jiménez-Bermúdez et al. 2002), with the highest reduction in softening being shown to occur during the transition from the white to the red stage. Within the gene families of cell wall-degrading genes of climacteric fruit, some members are regulated by ethylene, while others are not, confirming the coexistence of ethylene-dependent and -independent processes (Flores et al. 2001; Nishiyama et al. 2007). In general, it appears that fruit softening involves many genes that encode a variety of cell wall-degrading enzymes and non-enzymatic proteins. Each protein, and each protein isoform, may play a specific role in softening and textural changes.

Pigments are essential for the attractiveness of fruits, accumulating most often in the skin during the ripening process, although many climacteric fruits accumulate pigments also in their pulp tissue. The most important pigments of fruit are carotenoids and anthocyanins. Beside their role in pigmentation, they are important for human health as a source of vitamin A and antioxidant compounds. Carotenoids comprise carotenes, such as lycopene and β-carotene, and xanthophylls, such as lutein. They are derived from terpenoids, and are synthesized in fruit at a high rate during the transition from chloroplast to chromoplast. Many genes involved in the biosynthesis of carotenoids have been cloned (Cunningham and Gantt 1998; Hirschberg 2001), and extensive information is available on the regulation of carotenoid formation during fruit ripening (Bramley 2002). Anthocyanins belong to the flavonoid subclass of phenolic compounds. The flavonoid biosynthetic pathway has been elucidated in plants, and many enzymes and corresponding genes have been isolated and characterized (Winkel-Shirley 2001). In grape, where anthocyanins are crucial for the quality of wine, it has been demonstrated that ethylene (or the ethylene generator ethephon) stimulates berry coloration, demonstrating that this hormone is involved in the regulation of anthocyanin biosynthesis genes (El-Kereamy et al. 2003). A number of factors and signals influence the accumulation of anthocyanins and the expression of related genes,

including photochrome and light, hormones (gibberellins, methyl jasmonate), and various stresses such as wounding and low temperature (Mol et al. 1996). Environmental conditions and orchard management, including irrigation, pruning, and fertilization, are also known to strongly impact on fruit coloration.

Aroma volatiles contribute strongly to the overall sensory quality of fruit and vegetables. Extensive studies have been focused on the identification of volatile compounds, and to the elucidation of some of the biosynthetic routes either by bioconversion or by tracing of precursors (Sanz et al. 1997; D'Auria et al. 2002; Dudareva et al. 2004). In recent years, research efforts have been directed toward the isolation of the corresponding genes in fruits and vegetables (Aharoni et al. 2000; Yahyaoui et al. 2002; Beekwilder et al. 2004; El-Sharkawy et al. 2005; Pech et al. 2008b). Aroma is generally a complex mixture of a wide range of compounds. Each product has a distinctive aroma, which is function of the proportion of the key volatiles, and the presence or absence of unique components. The most important classes of aromas are monoterpenes, sesquiterpenes, and compounds derived from lipids, sugars, and amino acids. Ethylene is known to control the rate of ripening, the duration of storage life, and most of the ripening events in climacteric fruit. Therefore, breeders have "incidentally" reduced ethylene synthesis or action by generating genotypes with extended shelf life. Because many genes of aroma biosynthesis are ethylene-regulated (El-Sharkawy et al. 2005; Manriquez et al. 2006), this has often resulted in a severe loss of flavor in long-keeping genotypes that have commonly been generated by breeding with non-ripening mutants (McGlasson et al. 1987; Aubert and Bourger 2004). One major challenge for the future is to uncouple the down-regulation of ethylene from inhibition of aroma volatile production.

16.5 Molecular Markers and QTL Mapping of Fruit Ripening Traits

The advent of genetic approaches based on quantitative trait loci (QTLs) opens new prospects toward genetic improvement of fruit. Indeed, most fruit quality traits are under multigenic control, and the QTL approach allows the localization on genetic maps of loci responsible for at least part of the phenotypic variation, and enables the quantification of their individual effects. Because of the low molecular polymorphism observed in cultivated tomato, which is usually used as model species in fruit research, the majority of these studies (Tanksley and McCouch 1997; Causse et al. 2002) rely on interspecific progeny. Surprisingly, in spite of their characteristics inferior to those of cultivated species, wild species can possess alleles useful for improving fruit traits. A good example is given by a QTL improving fruit color, detected in a *Solanum habrochaites* (*Lycopersicon hirsutum*), a green-fruited species. The molecular markers localized in the vicinity of this QTL are now being used in marker-assisted selection to create parent lines with increased potential, or in contrast, to avoid certain unfavorable traits (Fulton et al. 2002). A fruit weight QTL, common to several studies, has been precisely localized and then cloned by

chromosome walking (Frary et al. 2000). Another QTL controlling sugar concentration in fruit has also been cloned (Fridman et al. 2000), and the gene responsible for this QTL has been shown to encode a cell wall invertase (Fridman et al. 2004).

As emphasized above, the climacteric character represents an important determinant of the ripening rate and storability. Because genetically compatible climacteric and non-climacteric types of melon are available, it has been possible to study the inheritance of the climacteric character. A segregating population resulting from a cross between a typical climacteric-type Charentais melon (*Cucumis melo* var. *cantalupensis* cv. *Védrantais*) and a non-climacteric melon, Songwhan Charmi PI 161375 (*Cucumis melo* var. *chinensis*), has been generated and used to study the segregation of the formation of the abscission layer (*Al*) of the peduncle and ethylene production (Périn et al. 2002). It was found that the climacteric character was controlled by two duplicated independent loci (*Al-3* and *Al-4*), and the intensity of ethylene production was controlled by at least four QTLs localized in other genomic regions. None of the QTLs matched with known genes of the ethylene biosynthetic or transduction pathways. Recently, it was reported that some introgression lines generated from two non-climacteric melons, Piel de Sapo (var. *inodorus*) and Songwhan Charmi PI 161375 (var. *chinensis*) possessed a climacteric character (Obando et al. 2007). The QTLs associated with ethylene production and respiration rate in this work have not been mapped at the same position as the *Al* loci described by Périn et al. (2002). Collectively, these data suggest that different and complex genetic regulation exists for the climacteric character.

Improvement of fruit quality arose in some cases randomly, like in the apple, where a chance seedling, Golden Delicious, was discovered with good agronomic characters. It has been crossed with old apple varieties having good sensory attributes to generate new apple cultivars that combine good agronomic and good sensory characters (Vaysse et al. 2000). Similarly, the poor-keeping qualities of Delicious have been improved by crossing with long-keeping apples (Rall's Janet), giving rise to the Fuji group of apples (Vaysse et al. 2000). Likewise, in Charentais-type melons, long or mid-shelf life commercial genotypes are available. Some of these have been generated using a non-ripening melon named "Vauclusien". However, the long shelf life character is often associated with poor sensory qualities (Aubert and Bourger 2004). Low ethylene production is generally correlated with long storage life. The delayed ripening of these genotypes was found to result in alteration of ethylene biosynthetic or response genes. The amount of ethylene in ripening Fuji apples parallels the transcript levels of the ripening-specific *ACS* gene, *MdACS1* (Harada et al. 1985). An allele of this gene (*MdACS1-2*) contains an insertion of a retro-transposon-like sequence in the 5′-flanking region, and is transcribed at a lower level than the wild-type allele *MdACS1-1*. Cultivars that are homozygous for the *MdACS1-2* allele have low ethylene production and long storage life (Sunako et al. 1999). Two *ERF* genes (*MdERF1* and *MdERF2*) have been isolated from ripening apple fruit. The *MdERF1* gene has been shown to express predominantly in ripening fruit, and *MdERF2* exclusively in ripening fruit (Wang et al. 2007). Expression of both genes was repressed by treatment with 1-MCP. Apple cultivars with low ethylene production had a tendency to show lower

expression of these two *MdERF* genes than those with high ethylene production. By screening different cantaloupe melons, Zheng and Wolff (2000) reported a correlation between ethylene production and post-harvest decay. In addition, using *ACO* cDNA probes, they were able to demonstrate that low ethylene production was associated with the presence of an RFLP *ACO* allele *Ao*, whereas high ethylene production was associated with the *Bo* allele in homozygous conditions (Zheng et al. 2002).

Amongst climacteric fruits, there are genetic differences in the capacity to induce the ripening process. The most striking case is given by fruit cultivars that require exposure to post-harvest low temperatures for ripening. Some winter pear varieties, such as D'Anjou, Beurre Bosc, and Passe Crassane, require chilling temperatures for the induction of autocatalytic ethylene production (Blankenship and Richardson 1985; Morin et al. 1985; Knee 1987). Furthermore, it has been reported that the cold-requirement character can be transmitted by breeding, as exemplified by crossing of Passe-Crassane pears and a cold-independent variety, Old Home, to give a mixed population of cold-dependent and cold-independent hybrids (El-Sharkawy et al. 2004). Cold requirement appears to be linked to the possibility of inducing ethylene biosynthesis genes. In Passe Crassane pears, a 3-month chilling treatment at 0°C strongly stimulated ACC oxidase activity, and to a lesser extent, ACC synthase activity (Lelièvre et al. 1997). It has been shown that the presence of some *ACS* alleles was correlated with the chilling requirements for ripening, and with the induction of autocatalytic ethylene production (El-Sharkawy et al. 2004).

16.6 Natural Mutants Affected in the Ripening Phenotype

Among the reasons why tomato has emerged as a model species for studying fleshy fruit development is the presence of well-characterized, spontaneous mutants or wild-allele variants that have been recovered from production fields or breeding programs. A number of genes corresponding to various mutations have been isolated by positional cloning (Giovannoni 2007). The first ripening-impaired mutant to be characterized at the molecular level is *Never-ripe* (*Nr*), which bears a dominant mutation that affects the ethylene response, and results in fruit producing reduced amounts of ethylene and retaining very low ethylene responsiveness (Lanahan et al. 1994). It was shown that the *NR* gene encodes an ethylene receptor from the ERS family devoid of receiver domain (Wilkinson et al. 1995). The *Green-ripe* (*Gr*) mutant corresponds also to a dominant ripening mutation lying in a gene encoding a new component of ethylene signaling (Barry and Giovannoni 2006), corresponding to the *Reversion To Ethylene Sensitivity1* (*RTE1*) shown to interact and regulate the ETR1 ethylene receptor in Arabidopsis (Resnick et al. 2006; Zhou et al. 2007).

One of the tomato mutations most commonly used by the breeders affects the transcriptional control of fruit ripening. The *ripening-inhibitor (rin)* mutation is a recessive mutation that blocks the ripening process, and prevents ethylene

production and responsiveness. In the last decade, the *rin* locus has been widely used for generating long shelf life commercial varieties. The *rin* mutation encodes a MADS box-type transcription factor that is present in both climacteric and non-climacteric fruit (Vrebalov et al. 2002), suggesting that it probably acts upstream of the climacteric switch. The *Colorless non-ripening* (*Cnr*) mutant is a dominant mutant corresponding to an epigenetic mutation that alters the methylation of the promoter of a SPB box transcription factor (Manning et al. 2006). Although it has been proposed that both *rin* and *cnr* act upstream of ethylene production (Giovannoni 2007), the location of these two transcription factors in the ripening regulatory network is not clear.

A number of other mutants affect the fruit composition in terms of secondary metabolites. Because of the ease of visual screening, most of the mutants affected in fruit composition are altered in pigment accumulation. The color change from green to red associated with ripening in tomato results from both chlorophyll degradation and carotenoid pigment accumulation. The numerous tomato mutants affected in pigmentation represent a valuable genetic resource, which has been exploited to facilitate the identification of the genes involved in carotenoid biosynthetic pathways, and understanding the complex mechanisms regulating pigment accumulation (Bramley 2002). The role of light has been reported in the regulation of fruit pigmentation (Giovannoni 2001). The *yellow-flesh* (*r*) mutation that results in the absence of carotenoid accumulation corresponds to a deletion within the ethylene-regulated *phytoene synthase-1* gene (Fray and Grierson 1993). The *delta* mutant displays an orange color resulting from the accumulation of δ-carotene at the expenses of lycopene (Tomes 1969), due to a dominant mutation within the *CrtL-e* gene encoding a lycopene ε-cyclase (Ronen et al. 1999). The *Beta* (*B*) partially dominant mutation also results in orange color, due to the accumulation of β-carotene instead of lycopene. The gene responsible for the *B* mutation encodes a fruit- and flower-specific lycopene, β-cyclase, capable of converting lycopene into β-carotene. Its expression is strongly increased in the *B* mutant (Ronen et al. 2000). Deep-red fruit of *old-gold* and *old-gold-crimson* mutants are null mutations of an allele of the B gene (Ronen et al. 2000). *Tangerine* is a recessive mutation conferring orange color by accumulation of pro-lycopene instead of normal lycopene. It corresponds to an impairment of the expression of a carotenoid isomerase gene that is suspected to enable carotenoid biosynthesis in the dark, and in non-photosynthetic tissues (Isaacson et al. 2002). The *hp1* and *hp2* mutants exhibiting elevated content of flavonoid and carotenoid are mutated in *Damaged DNA Binding Protein1* (Liu et al. 2004), and *Detiolated1* (Mustilli et al. 1999) genes, respectively. The corresponding genes in Arabidopsis encode nuclear-localized light signal transduction proteins.

16.7 Conclusions and Future Directions

While tremendous progress has been made in understanding the mechanisms of fruit ripening, a number of questions remain unanswered. In climacteric fruit, amongst the major issues that remain to be addressed are the role of hormones

other than ethylene, and the way in which they interact with ethylene signaling to control different aspects of fruit ripening. The mechanism by which ethylene selects specific ripening-regulated genes is another important topic that needs to be investigated. In non-climacteric fruit, the detailed mechanisms that regulate the ripening process remain largely unknown, although molecular data are accumulating. So far, the regulation of gene expression during fruit ripening has been viewed mostly at the transcriptional level. Recent studies on the ethylene receptor (Kevany et al. 2007) illustrate that post-transcriptional regulation plays an essential role, and deserves more attention. Regulation of gene expression by epigenetic variations is now recognized as an important determinant of plant development. Epigenetic variations do not affect the primary DNA sequence, but consist of DNA methylation or histone modifications that affect gene expression generally at the level of chromatin organization. In fruit, the *Cnr* mutation is the only well-characterized, natural and stably inherited epigenetic mutation (Seymour et al. 2007). Research efforts are now being directed toward understanding the epigenetic regulation of the fruit ripening process. It is predictable that the answers to these questions will require cooperation between fruit physiologists, molecular biologists, and geneticists.

The recent development of high-throughput technology for analyzing genome structure and functions is starting to have an impact on fruit research. A number of national and multinational programs are attempting to combine genomics, proteomics, metabolomics, and reverse genetic approaches to unravel the molecular mechanisms of fruit development (Wang et al. 2009). The implementation of these genome-wide (Alba et al. 2004) and metabolomic technologies (Overy et al. 2005), together with bioinformatics tools, is expected to provide new understanding of the fruit developmental program, and reveal the networks of interactions between different pathways leading to the accumulation of fruit quality traits. The most important programs are being implemented on the tomato model species. A multinational consortium has been established recently, which has made available centralized facilities for tomato ESTs and derived DNA chips (Mueller et al. 2005). This is enabling the elucidation of global changes in gene expression during fruit development and ripening, and researchers to mine and analyze the expression profiling data in order to cluster the complete set of genes involved in specific metabolic and regulatory mechanisms. By comparing differences between natural variants, ripening mutants, or introgression lines, genes will be identified that are essential for specific aspects of fruit ripening, with their corresponding impact on fruit metabolism (Fei et al. 2004; Overy et al. 2005). In addition, reverse genetics approaches for high-throughput functional identification of target genes are being developed, amongst which the emerging TILLING (targeting induced local lesions in genomes) technology is most promising. The completion of the tomato genome sequencing project, and the availability of the tomato genome sequence in the near future will represent a major breakthrough likely to change our understanding in the area of the fundamentals of fruit growth and development, and open new avenues to address the varied topics in fruit research.

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Chapter 17
Seed Development

B. Dubreucq, S. Baud, I. Debeaujon, C. Dubos, A. Marion-Poll, M. Miquel, H. North, C. Rochat, J.-M. Routaboul, and L. Lepiniec

17.1 Introduction

Seed development and maturation represent an evolutionary advantage allowing most plants to cope with unfavourable environmental conditions by interrupting their life cycle and resuming growth when placed under favourable conditions (Bewley 1997; Bentsink and Koornneef 2002). Seeds are a major source of nutrients for animals and humans, and knowledge of their biology is essential for the improvement of agricultural practices and the management of genetic resources. Seeds accumulate large amounts of storage compounds such as carbohydrates, oils and proteins (seed storage proteins, SSPs). The main metabolic pathways necessary for their accumulation are well characterized and, for agro-industrial purposes, it would be interesting to engineer seeds that accumulate greater concentrations of selected compounds. Indeed, there is tremendous interest in using seeds as a sustainable alternative to fossil reserves for green chemistry. The production of secondary metabolites in seeds is also a key objective as these compounds possess industrial and therapeutic potential. Since the overall regulation of the different pathways is still unclear, the understanding of the genetic control of seed development and metabolism remains an important area of research.

B. Dubreucq, S. Baud, I. Debeaujon, C. Dubos, A. Marion-Poll, M. Miquel, H. North, C. Rochat, J.-M. Routaboul, and L. Lepiniec
Institut Jean-Pierre Bourgin (IJPB), Seed Biology Laboratory, UMR 204 INRA/AgroParisTech, 78026 Versailles Cedex, France
e-mail: lepiniec@versailles.inra.fr

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17.2 The Use of a Model Plant for the Study of Embryo Development and Maturation

17.2.1 Embryo Development

Arabidopsis thaliana is a widely used model that is very suitable for molecular genetics. Its seed development and maturation are well documented (Baud et al. 2002; Weijers and Jurgens 2005; Lepiniec et al. 2005) and can be extended to other plant models and crops. Embryo morphogenesis is initiated by double fertilization in the embryo sac, giving rise to the endosperm and the zygote. The zygote divides asymmetrically to form apical and basal cells that lead to the embryo proper and the suspensor respectively. A precise order of events ensures the correct relative positioning of the different tissues and organs (meristems, cotyledons and hypocotyl) and the arrangement of cell types within each tissue of the embryo. At the heart stage of embryo development (about 7 days after fertilization, "daf"), most of the cell division and differentiation events have already occurred, i.e. the protoderm has differentiated into the epidermis, the pro-vascular bundles are ready to form the vascular system, and the overall shape of the embryo is determined with the organization of both apico-basal (shoot and root meristems) and lateral (cotyledons) symmetries (Fig. 17.1 left panel). During this first step, the triploid endosperm develops with an initial syncytial phase followed by cellularization and differentiation events (Berger et al. 2006).

17.2.2 Embryo Maturation

During the maturation phase, embryo growth and cell cycle activities stop (Raz et al. 2001). The embryo goes through a period of cellular expansion and differentiation, concomitant with the reduction of the endosperm to one cell layer and the onset of maturation. Seed maturation is characterized by storage compound accumulation and the acquisition of tolerance to desiccation. In seeds of *A. thaliana*, triacylglycerols (TAGs) and storage proteins (SSPs) constitute the main storage compounds. To synthesize these storage products, the developing seed imports assimilates from the mother plant via phloem strands. In rapeseed, for instance, phloem sap is composed mainly of sucrose and amino acids such as glutamate (Glu), glutamine (Gln) and serine (Ser; Lohaus and Moellers 2000). These nutrients are unloaded from maternal tissues, released into the apoplastic space and, finally, loaded into zygotic tissues (Ruuska et al. 2002; Zhang et al. 2007). Once in the embryo, incoming sucrose is rapidly metabolised through the glycolysis and/or the oxidative pentose phosphate pathway. By means of a set of complementary techniques, such as microarray experiments and metabolic flux analyses using ^{13}C labelled metabolites, Ruuska et al. (2002) and Schwender et al. (2003) have provided a comprehensive overview of central carbon metabolism in maturing oilseeds.

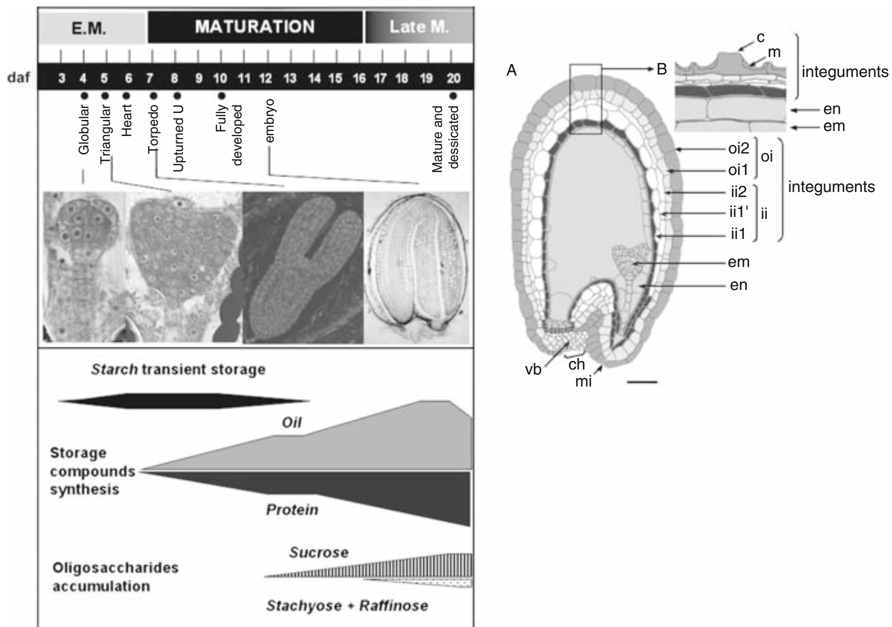

Fig. 17.1 *Left panel* Arabidopsis seed development (from Baud et al. 2002): early morphogenesis (E.M.) to maturation and late maturation (late M.). Accumulation profiles of storage compounds are indicated *below*. *Right panel* Arabidopsis seed coat anatomy. *A* Longitudinal section of an immature seed (scale bar = 65 μm). *B* Structure of the mature dry seed coat (magnified 3× compared to the region indicated in *A*. The cell layers ii1 (also called endothelium), ii2 and oi2, which synthesize condensed tannins, flavonols or mucilage during seed coat (testa) differentiation, are indicated in *black*, *light grey* and *dark grey* respectively. Abbreviations: daf, days after flowering; c, columella; ch, chalaza; em, embryo; en, endosperm; ii, inner integument; m, mucilage; mi, micropyle; oi, outer integument; vb, vascular bundle

17.2.2.1 Starch

In several crop species like *Pisum sativum*, *Vicia faba*, wheat and maize, starch constitutes an important storage product and the corresponding biosynthetic pathway is well documented. Briefly, glucose-6-phosphate (Glc-6-P) is transported from the cytosol to the plastid and transformed into Glc-1-P by phosphoglucomutase. ADP-glucose pyrophosphorylase then catalyses the formation of ADP-glucose, the constituent of both amylose and amylopectin (Zeeman et al. 2002). Interestingly, starch is only transiently accumulated in oilseeds (King et al. 1997) and its role in these seeds is highly debated (Vigeolas et al. 2004).

17.2.2.2 Oil

Triacylglycerols (TAGs) are synthesized by the sequential acylation of three fatty acids at positions *sn*-1, *sn*-2 and *sn-3* of a glycerol-3-phosphate moiety, and

are then stored in dedicated organelles, the oil bodies. During recent years, substantial efforts have been made to either modify oil fatty acid composition or increase yield, bringing a wealth of knowledge about the different steps of TAG biosynthesis. Nonetheless, much remains to be determined about the mechanisms controlling fatty acid chain length, removal of fatty acids from their site of synthesis before modification, exclusion of unusual fatty acids from membrane lipids and incorporation into TAGs. This knowledge is critical for successful metabolic engineering in crop species of foreign fatty acids (Graham et al. 2007; Dyer and Mullen 2008). Some examples of important targets for biotechnological approaches are presented here.

In the plastid, fatty acid biosynthesis proceeds by two-carbon elongation steps catalysed by a small family of β-ketoacyl-acyl-carrier protein (ACP) synthases, often referred to as condensing enzymes of the β-ketoacyl-acyl carrier protein synthase (KAS) family. In wild-type seeds of *A. thaliana*, the KASII enzyme converts most of the 16:0-ACP formed to 18:0-ACP. However, 16:0-ACP is also the substrate for competing enzymes such as plastidial lysophosphatidic acid acyltransferase (Kim and Huang 2004), Δ^9 acyl-ACP desaturase (Cahoon and Shanklin 2000), and the acyl-acyl protein thioesterase B1 (FATB1) that hydrolyses the 16:0-ACP prior to export of the corresponding free fatty acid outside the plastid (Bonaventure et al. 2003). Reducing KASII expression in a tissue-specific manner was recently shown to increase 16-carbon fatty acid accumulation in TAGs, thereby converting the composition of a temperate seed oil into that of a palm-like tropical oil (Pidkowich et al. 2007).

Once fatty acids have been exported outside the plastids and activated to acyl-CoA esters, they can be elongated further in the cytosol to form very long chain fatty acids or, once acylated, modified into membrane phospholipids. Modifying enzymes like fatty acid desaturases (FADs) and so-called divergent FADs found in non-domesticated plant species are capable of producing many different types of industrially valuable fatty acids (Drexler et al. 2003). The cDNAs encoding such enzymes have been cloned and transferred into crop species. Studies of the corresponding transgenic lines have shown the complexity of the fatty acid biosynthetic machinery and the need for these exotic fatty acids to be excluded from membranes because of their physicochemical properties. Efforts are now being focused on identifying and cloning both acyltransferases and enzymes contributing to microsomal channelling of acyl groups into TAGs by exclusion and/or by removal ("editing") of toxic fatty acids from membrane lipids (Drexler et al. 2003; Dyer and Mullen 2008).

Measurements of glycerol-3-phosphate (Gly-3-P) levels in developing seeds have shown that the rate of Gly-3-P supply could restrict TAG accumulation. This was confirmed by feeding maturing seeds with exogenous glycerol (Vigeolas and Geigenberger 2004). Gly-3-P can be synthesized by phosphorylation of glycerol by glycerol kinase or, alternatively, by oxidoreduction of dihydroxyacetone phosphate by a NAD^+:glycerol-3-phosphate dehydrogenase (Gly3PDH). The involvement of cytosolic Gly3PDH isoforms in the production of glycerol moieties for TAG biosynthesis was recently demonstrated using a transgenic approach.

The overexpression in *Brassica napus* of a yeast gene coding for cytosolic Gly3PDH (*gpd1*) enhanced Gly3PDH activity, led to a strong increase in Gly3P levels in seeds, and resulted in a 40% increase in the final lipid content of seeds (Vigeolas et al. 2007).

17.2.2.3 Storage Proteins

SSPs are synthesized in the endoplasmic reticulum and accumulated in protein storage vacuoles (PSVs). In *A. thaliana* seeds, two predominant classes of SSPs are stored: legumin-type globulins, referred to as 12S globulin or cruciferin, and napin-type albumins, referred to as 2S albumin or arabin. Both types of SSPs are synthesized as apoproteins that are transported by a vesicle-mediated pathway and converted into their mature forms by limited proteolysis upon arrival at the PSVs. Processing of SSPs involves both Asn-specific endopeptidases (or vacuolar processing enzymes, VPEs) and aspartic proteases. The complete removal of VPE activity in a quadruple mutant of *A. thaliana* results in the accumulation of alternatively processed forms, demonstrating that maturing seeds can tolerate variations in the protein content of PSVs (Gruis et al. 2004). Several genetic engineering strategies have been developed in *A. thaliana* and in crop species to increase and/or modify the protein content of seeds. For instance, enhancing the transport of amino acids from source to sink by overexpressing genes for Asn synthase or amino acid transporters increased the amount of endogenous seed proteins, illustrating how the SSP content is correlated to amino acid supply (Lam et al. 2003; Rolletschek et al. 2005). Since essential amino acids like lysine (Lys) or methionine (Met) contribute significantly to the nutritional quality of seeds, biotechnological approaches have been used to improve the amount of these compounds in seeds. The in planta overexpression of bacterial dihydrodipicolinate synthases, which are less sensitive to Lys inhibition than is the case for their plant counterparts, doubled the levels of free Lys in seeds. However, these transgenic seeds exhibited poor germination rates, and alternative strategies such as the expression of modified or exogenous SSPs rich in Lys (or Met) have been developed to circumvent this problem (Shewry 2007).

17.2.2.4 Regulation of Storage Compound Synthesis

The sucrose-to-hexose ratio is known to promote the synthesis of storage compounds in legume seeds, although the molecular regulatory mechanisms involved have not been fully elucidated to date (Weber et al. 2005). The importance of this ratio in the control of the switch to storage product accumulation in oleaginous species remains controversial. Trehalose-6-phosphate is also thought to play a crucial role in developing *A. thaliana* embryos, but the regulatory mechanism involved is unknown (Gomez et al. 2006).

17.3 The Genetic Control of the Embryo Maturation Phase

The maturation processes occurring in various seed tissues (i.e. seed coat, endosperm and embryo) contribute to seed quality, promoting efficient dispersal and seedling establishment (Welbaum et al. 1998). Seed quality relies, therefore, on the tight control of embryo morphogenesis, maturation and germination. Several observations suggest that the different processes can be disconnected to some extent. Various mutants affected in embryogenesis can still express seed maturation-specific genes, and accumulate some storage compounds (Lepiniec et al. 2005). Nevertheless, simple genetic switches may exist for shifting from embryogenesis to a maturation program and, subsequently, to germination. These genetic programs may be exclusive at the cellular level but could occur simultaneously in the same embryo. The nature and origin of the molecular mechanisms controlling both the entry into the maturation phase and the prevention of cell growth and division remain to be elucidated.

17.3.1 Transcriptional Regulation

Transcriptional regulation plays a central role in the regulation of the coordinated and successive phases of seed development. *LEC2*, *FUS3* and *ABI3* encode related transcription factors of the "B3-domain" family (Giraudat et al. 1992; Luerssen et al. 1998; Stone et al. 2001). This domain shares some similarities with a prokaryotic DNA-binding-domain endonuclease. Belonging to a different class of proteins, LEC1 is homologous to HAP3 subunits of the CAAT box-binding factors (CBFs), a family of heteromeric transcription factors (Lee et al. 2003). *LEC1* and *LEC2* are specifically expressed in seeds, early during embryogenesis (Stone et al. 2001; Lee et al. 2003). The expression of *FUS3* has been detected mainly in the protodermal tissue of the embryo (Tsuchiya et al. 2004). This L1-specific expression pattern is difficult to reconcile with a direct effect of FUS3 on the promoter of *SSP* genes throughout the embryo. Recent data have shown that *ABI3*, although detected in vegetative organs, is expressed throughout the embryo, consistently with the expression pattern of its target genes (To et al. 2006).

Mutations of *ABI3* (Koornneef et al. 1984), *LEC1* (Meinke 1992), *LEC2* (Meinke et al. 1994) and *FUS3* (Baumlein et al. 1994) genes lead to similar pleiotropic seed phenotypes (Parcy et al. 1997; Harada 2001). Mutant cotyledons display some of the characteristics of young leaves, exhibiting both trichomes on their surface and a complex vascular pattern. The mutant embryos can also present abnormal suspensor phenotypes, precocious cell cycle activation, and growth of apical and root meristems. Mutant seeds are less tolerant to desiccation, accumulate less storage compounds, and accumulate anthocyanin pigments and/or are affected in chlorophyll breakdown. When placed in a humid environment, they can display precocious germination, and mutant combinations show extreme vivipary.

Furthermore, some specific phenotypes appear to be additive in double mutants, suggesting that the three proteins belong to a complex network of local and redundant pathways that partially overlap (To et al. 2006). These genetic analyses are supported by various molecular datasets.

Ectopic expression of *LEC1* induces embryo development in vegetative cells (Lotan et al. 1998), and the induction of *LEC2* in maturing embryos correlates well with the onset of oil deposition. Moreover, the ectopic expression of *LEC2* in developing leaves was shown to be sufficient to trigger TAG accumulation in these tissues (Santos Mendoza et al. 2005). Recent advances have been made in the elucidation of the transcriptional regulation of the actors participating in the biosynthesis of storage compounds in *A. thaliana*. The *WRINKLED1* gene, a direct target of LEC2, encodes a transcription factor of the AP2-EREBP family and appears to specify the regulatory action of LEC2 towards the fatty acid biosynthetic pathway by triggering the expression of genes encoding enzymes of the glycolysis and the fatty acid biosynthetic machinery (Baud et al. 2007). Similarly, it has been shown that the ectopic expression of *FUS3* can trigger the expression of fatty acid biosynthetic genes (Wang et al. 2007). In *A. thaliana*, the recent discovery that the overexpression of *GmDof4* and *GmDof11*, two Dof-type transcription factors from soybean, resulted in the induction of genes involved in fatty acid biosynthesis whilst repressing *CRA1*, an SSP gene, may pave the way to understanding how the protein-to-oil ratio is modulated in seeds.

Other important regulators of seed development and/or maturation have been identified by genetic analyses. Several bZIP transcription factors have been shown to play a role during seed development (Finkelstein and Lynch 2000; Lopez-Molina and Chua 2000). These transcription factors act together with ABI3 to regulate the expression of *SSP* genes (Lara et al. 2003). The bZIP ABI5 protein binds to ABRE *cis* elements present in the promoters of several late embryogenesis abundant (LEA) genes such as *AtEM1* or *AtEM6*. Interestingly, PICKLE (PKL), a chromatin remodelling factor (CHD3), acts in concert with GA (gibberellic acid) to repress embryonic traits during and after germination, including the expression of *LEC1*, *LEC2* and *FUS3* in roots (Ogas et al. 1999; Henderson et al. 2004; Li et al. 2005). Some reports suggested that epigenetic mechanisms could repress the expression of seed-specific genes during seed germination (Tai et al. 2005) by controlling the activation of maturation-specific genes (Ng et al. 2006) or histone acetylation processes (Tanaka et al. 2008).

17.3.2 Control of Target Gene Expression

It has been shown that B3-type transcription factors can act directly on the expression of genes encoding storage proteins (Kroj et al. 2003; Vicente-Carbajosa and Carbonero 2005; Braybrook et al. 2006). The three B3-type regulatory proteins can directly activate target genes in vivo and in vitro by binding to RY motifs present in the promoters (Monke et al. 2004). Indeed, the “RY” box (CATGCA) is necessary

for the correct expression of several seed-specific genes in, for instance, *A. thaliana*, legumes (Dickinson et al. 1988) and maize (Suzuki et al. 1997). However, RY elements are probably not recognized alone in seed promoters. It has been shown that other DNA motifs (e.g. "G box") and factors binding these motifs are required for the correct expression of target genes during seed maturation (Wobus and Weber 1999; Ezcurra et al. 1999; Bensmihen et al. 2002; Vicente-Carbajosa and Carbonero 2005).

17.4 Seed Coat Development and Differentiation

The seed coat (testa) is a maternal tissue surrounding the embryo and the residual endosperm. It consists of the chalazal tissues, where the vascular bundle connecting the seed to the mother plant ends, and of the integuments that develop and differentiate from ovular integuments upon fertilization. As indicated in Fig. 17.1 right panel, *A. thaliana* seeds are bitegmic (cf. two integuments), the inner integument having three cell layers in most of the seed coat, except at the micropylar pole (two layers), and the outer integument being two-layered (Haughn and Chaudhury 2005). In the course of seed development and maturation, the testa undergoes important modifications, from growth through both cell division and expansion in early stages (Haughn and Chaudhury 2005), to death and dramatic compression of cell layers in the mature testa (Fig. 17.1 right panel). Crosstalk between the female gametophyte, ovule/seed integuments and endosperm has been shown to be important for the control of seed size, as well as seed coat development and differentiation in *A. thaliana* (Ingouff et al. 2006). Yet, any role of the *A. thaliana* seed coat in embryo nutrition remains obscure.

17.4.1 Structure of the Integuments

The five integumentary cell layers have different fates. The parenchymatic ii1 and ii2 layers undergo early cell death involving a cysteine proteinase with caspase-like activity (Nakaune et al. 2005). Proanthocyanidins (condensed tannins) are polymeric flavonoids that are present specifically in the innermost integumentary cell layer or endothelium (ii1 layer) in *A. thaliana* (Debeaujon et al. 2003; Routaboul et al. 2006). These secondary metabolites accumulate in vacuoles as colourless compounds during the first 5–6 daf and become oxidized into brown pigments by the laccase-type polyphenol oxidase TRANSPARENT TESTA (TT) 10 during seed desiccation (Pourcel et al. 2005, 2007). The oi1 subepidermal cell layer undergoes secondary thickening of the inner tangential cell wall (Beeckman et al. 2000). It also accumulates colourless to pale yellow flavonoids called flavonols that are present mainly as glycoside derivatives not only in the testa but also in the embryo and endosperm (Pourcel et al. 2005; Routaboul et al. 2006).

Many functions involved in flavonoid metabolism (biosynthesis, regulation, trafficking) have been identified by a mutant approach based on visual screening for changes in seed coat pigmentation (Lepiniec et al. 2006). The oi2 epidermal layer differentiates into external seed coat cells with thickened radial cell walls and central elevations, known as columella, surrounded by mucilage (Haughn and Chaudhury 2005). *A. thaliana* mucilage is composed primarily of the hydrophilic pectin domain rhamnogalacturonan I (Macquet et al. 2007b), and bursts out of the epidermal cells on imbibition to surround the seed. The released mucilage is composed of two layers with different physicochemical properties—an outer water-soluble layer, which can diffuse into the soil around the seed, and an inner adherent layer composed of two structurally distinct domains and that is tightly attached to the seed (Western et al. 2000; Macquet et al. 2007b). It has recently been shown that rhamnogalacturonan I structure is modified after its deposition in the apoplast by the removal of galactan/galactose ramifications (Dean et al. 2007; Macquet et al. 2007a). This alters mucilage hygroscopic properties, such that it swells sufficiently on imbibition to be released from oi2. A few regulatory genes important for the correct development of oi2 and several enzymes involved in mucilage biosynthesis have been identified (Western 2006). The chalazal region also undergoes changes, like PA biosynthesis in a few specific cells, as shown in Fig. 17.1 right panel (Debeaujon et al. 2003), and suberin deposition at the hilum (Beisson et al. 2007).

17.4.2 Regulation of Flavonoid Biosynthesis

In *A. thaliana*, structural genes required for flavonoid biosynthesis can be grouped into two categories, namely the early biosynthetic genes (EBGs: chalcone synthase, chalcone isomerase, flavonol 3-hydroxylase and flavonol 3′-hydroxylase), which are common to the different flavonoid sub-pathways (proanthocyanidins and flavonols), and the late biosynthetic genes (LBGs: flavonol synthase, dihydroflavonol-4-reductase, leucoanthocyanidin dioxygenase, anthocyanidin reductase), which include flavonoid transporters and decorating enzymes. It has been demonstrated that co-regulated genes have their expression controlled by a common set of regulatory proteins (Lepiniec et al. 2006). Such regulators have been identified in various species including maize, petunia, *Antirrhinum* and *A. thaliana*. Most of these regulators belong to the large R2R3-MYB and BHLH families of transcription factors. In *A. thaliana*, three regulators, namely TT2 (R2R3-MYB), TT8 (BHLH) and TTG1 (WDR), form a MYB-BHLH-WDR (MBW) complex that regulates the expression of the LBGs (including the TT12 transporter) leading to proanthocyanidin biosynthesis (Baudry et al. 2004, 2006). Three R2R3-MYB proteins (i.e. MYB11, MYB12 and MYB111), which are not part of a MBW complex, control flavonol biosynthesis through the regulation of EBGs and *FLS1* (*FLAVONOL SYNTHASE 1*; Stracke et al. 2007).

17.4.3 Biological Functions

The seed coat protects the enclosed embryo and endosperm against external biotic and abiotic stresses, thereby improving seed longevity. It also contributes to the control of seed germination and dormancy. The antioxidant, antibiotic, impermeabilizing and UV filtering properties of flavonoids contribute significantly to these biological roles (Debeaujon et al. 2000, 2007). In contrast, the physiological role of mucilage remains hypothetical (Macquet et al. 2007a).

17.5 Role of Phytohormones in the Control of Embryo Development and Seed Maturation

The key role of auxin in plant embryogenesis is well documented (Jenik and Barton 2005; Weijers and Jurgens 2005). Recent data suggest that auxin signalling may interfere with the "B3" regulatory network. Indeed, the ability of *lec1* and *lec2* mutants to form somatic embryos is strongly reduced, and auxin can induce *FUS3* expression (Gazzarrini et al. 2004). In addition, the role of *LEC1* in the promotion of embryonic cell identity and division has been shown to require auxin and sucrose (Casson and Lindsey 2006).

The precise role of abscisic acid (ABA) or gibberellic acid (GA) during embryo morphogenesis has not yet been demonstrated. In effect, mutants or transgenically modified plants either contain some traces of hormone or require its supply for normal and complete seed development (Phillips et al. 1997; Swain et al. 1997; Singh et al. 2002). Nevertheless, the relative concentrations of the hormones ABA and GA are critical for correct seed maturation and germination (Koornneef et al. 1982; Giraudat et al. 1994; Debeaujon and Koornneef 2000; Koornneef et al. 2002). It is important that growth of the embryo be arrested and premature germination inhibited by maintaining the amount of the germination-inducing hormone GA below a certain threshold relative to the levels of ABA. This is demonstrated by the production of viviparous seeds by double mutants affected in both embryo arrest (*fus3*, *lec1* or *lec2*) and ABA biosynthesis (Raz et al. 2001). Levels of ABA fluctuate during seed formation, with the major peak during seed maturation being mainly of maternal origin (Karssen et al. 1983). A second minor peak in ABA amounts observed during late maturation is synthesized by the zygotic tissues, embryo and endosperm, and this is required for dormancy induction (Karssen et al. 1983). In contrast, GA levels are relatively low during seed development, and increase only when seeds are imbibed (Ogawa et al. 2003). As well as inhibiting precocious germination, ABA produced during seed maturation is required for the accumulation of SSPs and LEA proteins, chlorophyll degradation, and acquiring desiccation tolerance and dormancy, as demonstrated by the phenotypes of severe *A. thaliana abi3* mutant alleles and transgenic tobacco that expressed antibodies to ABA (Ooms et al. 1993; Phillips et al. 1997). ABA

induces these different processes by activating either directly or indirectly a network of transcription factors. In particular, ABI3 has been shown to induce storage and LEA proteins through interactions with bZIP transcription factors such as ABI5 (Finkelstein et al. 2005). ABA signal transduction during seed maturation would also appear to be linked to metabolic homeostasis, as modification of the expression of a sucrose nonfermenting-1-related protein kinase in pea generated ABA insensitive seed maturation characteristics (Radchuk et al. 2006). Furthermore, transcriptome analyses in barley indicate that ABA signalling networks differ between the endosperm and embryo (Sreenivasulu et al. 2006). Maternal ABA can also affect the rate of seed development and seed coat maturation (Frey et al. 2004).

ABA is derived from carotenoid precursors synthesized in plastids, and the key regulatory step in its biosynthesis is the cleavage of epoxycarotenoids catalysed by 9-*cis*-epoxycarotenoid dioxygenase (NCED). This enzyme is encoded by a multigenic family in *A. thaliana*, with different members having specific spatiotemporal expression patterns (Tan et al. 2003), two of which are highly expressed in developing seeds (Lefebvre et al. 2006). The *AtNCED6* gene is expressed uniquely in the endosperm where it contributes to the synthesis of zygotic ABA, and *AtNCED9* is expressed in both embryo and endosperm; dormancy induction involves ABA synthesized in both zygotic tissues.

Active ABA levels depend not only on the concentrations of de novo synthesis but also on how much is catabolised. Several pathways exist for ABA deactivation involving either conjugation or hydroxylation (Nambara and Marion-Poll 2005). Phenotypes of mutants affected in three *CYP707A* encoding ABA 8′-hydroxylases have shown that degradation to phaseic acid plays a significant role in the modulation of ABA concentrations during seed development or in dry seeds (Okamoto et al. 2006). Despite the identification of ABA glucosyl transferases (Priest et al. 2006), the participation of ABA conjugation in the modification of ABA levels in seeds remains to be established. Nonetheless, conjugated ABA would appear to be produced in developing seeds, as some of the ABA present in dry seeds is produced from ABA glucose ester by action of a glucosidase AtBG1 (a β-glucosidase, AtBG1, which releases ABA from ABA glucose ester, is required for the production of some ABA present in dry seeds; Lee et al. 2006).

The regulation of ABA and GA concentrations during seed development appears interrelated. The expression of a number of GA biosynthesis genes is repressed by ABA during seed development, whereas genes that reduce the levels of bioactive GA are stimulated (Seo et al. 2006). This suggests that GA concentrations remain low during seed maturation in part due to the high concentration of ABA. The transcriptional regulator FUS3 has been found to control the spatiotemporal biosynthesis of both GA and ABA and these, in turn, regulate the stability of the FUS3 protein, thus forming a positive feedback loop with ABA synthesis during seed maturation (Gazzarrini et al. 2004). High ABA concentrations could, therefore, repress GA biosynthesis through FUS3. Similarly, ABA is required for the induction of some SSPs in seedlings ectopically expressing FUS3 (Kagaya et al. 2005) or ABI3 (Parcy et al. 1994). Conversely, it has been shown

that the activity of ABI3 and FUS3 can be regulated at the post-translational level by ABA and/or GA. The degradation of ABI3 can be triggered by AIP2 ("ABI3-Interacting Protein2"), an E3 ligase of which the expression is under the control of ABA (Zhang et al. 2005). This regulation could ensure a rapid degradation of ABI3 during imbibition, thereby promoting germination. Similarly, it has been suggested that ABA and GA could regulate the stability of FUS3 (Gazzarrini et al. 2004).

The importance of ABA in the control of seed maturation makes it an ideal candidate for the metabolic modification of certain seed traits such as storage protein accumulation, seed size and developmental rate. Transgenic *Nicotiana plumbaginifolia* mutants complemented for their ABA deficiency by the zeaxanthin epoxidase gene, under the control of a variety of seed specific promoters, did not show any differences in the timing of ABA biosynthesis; only the concentrations of ABA produced during seed maturation and concomitant effects on dormancy were observed (Frey et al. 2006). As conversion of the carotenoid zeaxanthin to violaxathin is an early ABA biosynthesis step, it is probable that downstream control mechanisms limited the effects of ectopic expression during seed development. To date, overexpression of *NCED* genes has been either through constitutive promoters or through induction of expression during imbibition (Thompson et al. 2000; Qin and Zeevaart 2002). Thus, it remains to be seen whether the temporal or spatial modification of ABA synthesis during seed maturation can be achieved. As transgenic plants ectopically expressing the ABA response factor ABI3 were induced to express transcripts encoding seed storage and desiccation tolerance proteins in vegetative tissues by ABA (Parcy et al. 1994), engineering the production or response to this hormone in seeds should be possible for the modification of important seed traits.

17.6 Conclusions

Seed production is of huge importance for human food and animal feed requirements. There is also a strong interest in using plant storage compounds for industrial applications (biomass for biofuels and biomaterials). For instance, plant oil is one of the most energy-rich and abundant forms of reduced carbon available in nature, and represents a possible substitute for conventional diesel. Thus, for nutritional, industrial as well as economical reasons, the accumulation of specific storage compounds still needs to be increased further.

Genetic engineering of the seed coat is also an attractive, although still underexploited, strategy to enhance seed quality and yield or to produce various metabolites and proteins (Moise et al. 2005). The feasibility of seed engineering has been demonstrated in maize kernels (Hood et al. 2007). The scale-up and diversified production of flavonoids as antioxidants for foods (Yilmaz 2006), human health promoting factors (e.g. nutraceuticals, pharmaceuticals; Ramos 2007), and mucilage as a source of pectin (Willats et al. 2006) are potential applications for seed coats as

"bioreactors". The improvement of cellulose fibre quality and yield from cotton testa trichomes (Lee et al. 2007), and the production of yellow-seeded *Brassica* genotypes with a greater meal nutritional value and oil content (Marles et al. 2003) are other crop breeding objectives where the seed coat is a focus of interest.

Interestingly, master seed regulators can directly control the accumulation of fatty acids, and SSPs and/or activate secondary transcription factors able to trigger other transcription programs. One can hypothesize that this complex network provides a robust and tight control of seed maturation. Various genetic and molecular analyses (using gain- and loss-of-function of different transcription factors) have demonstrated the feasibility of specifically and strongly increasing the metabolic flux of one particular sub-pathway through the synchronized regulation of the corresponding structural genes by transcription factors. This control of building-block accumulation may be easily combined with the expression of specific enzymes that modify the biosynthesis (e.g. accumulation and structure) of these compounds. However, our understanding of the gene regulatory networks that control seed development and maturation is still limited, since several transcription factors, their interactions, and their target genes remain to be fully characterized. In addition, other levels of regulation (e.g. post-translational or metabolic) will have to be taken into account for efficient metabolic engineering. Finally, the *Arabidopsis* model network will have to be extended to other plant species.

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Chapter 18
Seed Dormancy: Approaches for Finding New Genes in Cereals

J.M. Barrero, J. Jacobsen, and F. Gubler

18.1 Introduction

18.1.1 Dormancy and Adaptation

In their continuous struggle for survival in nature, plants have developed a vast array of strategies which allow them to adapt to the changing environment. Plants from different latitudes have evolved particular life cycles adapted to their location. Flowering time, bud sprouting, leaf senescence and seed germination are key traits enabling plants to grow and reproduce successfully in various ecological niches. To germinate or not to germinate may be the most important decision that plants have to make; germination must be optimized for environmental conditions which are best for the establishment of the new plant. Seeds of different species growing in different environments often have different requirements for germination and may lie dormant for long periods until these requirements are met (Bewley 1997; Baskin and Baskin 1998; Finch-Savage and Leubner-Metzger 2006).

Dormancy is an adaptive trait which seeds acquire during their development and maturation, and can be defined as the repressive state which temporarily blocks germination. A seed under this block is called dormant and, once dormancy is released, the seed is non-dormant. The dormancy initiated during seed maturation is primary dormancy, while secondary dormancy can be initiated in mature non-dormant seeds under unfavourable environmental conditions such as high temperatures. Different parts of the seed play roles in dormancy. Embryo dormancy is very important and is related mainly to attenuating growth potential. The seed coat, and the glumes of cereals, can act as physical barriers which limit

J.M. Barrero, J. Jacobsen, and F. Gubler
CSIRO Plant Industry, P.O. Box 1600 Canberra ACT 2601, Australia
e-mail: jose.barrero@csiro.au

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oxygen, water and nutrient uptake by the embryo as well as, perhaps, the egress of dormancy-promoting factors. The endosperm can act also as a barrier which restricts embryo growth in imbibed seeds. Dormancy loss can be affected by a number of environmental factors like light, temperature, nutrients, drought, after-ripening and fire. Plants have evolved different strategies to utilize dormancy to their advantage. Some plant species produce seeds which are non-dormant, while others produce seeds which are weakly or deeply dormant. Other plants can produce seeds with different degrees of dormancy, depending on environmental stimuli, and the dormancy can be maintained even if the external conditions are suitable for germination. Seed dormancy probably evolved as a mechanism for permitting germination when the environmental conditions become more conducive to growth and reproductive success. In some instances, this can result in seeds remaining dormant in soil for many years (reviewed in Bewley 1997; Baskin and Baskin 1998; Koornneef et al. 2002; Finkelstein et al. 2008).

18.1.2 Plant Domestication and Dormancy

Seed dormancy is widespread in the plant kingdom and is more common in species living at high latitudes (Baskin and Baskin 1998; Finch-Savage and Leubner-Metzger 2006). During the domestication of crops, plants have been modified to improve their agronomic characteristics, such as ease of harvest, germinability and flowering time. The characteristics of domesticated plants are known as the domestication syndrome (reviewed in Finch-Savage and Leubner-Metzger 2006). The selection of grains with no or low primary dormancy is a major feature of the domestication syndrome in cereal crops, and has been described as the first selected trait in the Neolithic age. Even in the model plant *Arabidopsis thaliana*, the more common laboratory ecotypes have low seed dormancy (e.g. Columbia), which may be due to selection for rapid germination. Similarly, many cereal cultivars display weak grain dormancy, which increases susceptibility to pre-harvest sprouting, a problem common to many cropping areas in the world, especially those affected by moist weather prior to harvesting (Gubler et al. 2005). Pre-harvest sprouting in wheat results in grains with high α-amylase activity which causes starch degradation, thus reducing the quality of the grain. Varieties with strongly dormant grains are free of this problem. On the other hand, an excessive level of dormancy can also be problematic, because the grains then need to be stored for several months or to be treated in order to break dormancy. This is especially important in the barley malting process, where grains need to germinate rapidly and uniformly (Gubler et al. 2005; Finch-Savage and Leubner-Metzger 2006). The control of seed dormancy, especially primary dormancy, is an important research area for cereal breeding.

18.1.3 A Complex Trait

Dormancy is currently being intensively investigated in many laboratories around the world. More than 1,500 publications with the topic "seed dormancy" have appeared in the last 5 years, including more than 50 reviews in scientific journals and books chapters (ISI Web of Knowledge, Thomson Scientific, USA; http://apps.isiknowledge.com). Seed dormancy is a very complex trait governed by a web of interactions between environmental and genetic factors (Fig. 18.1a). The major environmental factors affecting seed dormancy are temperature and light, but also nutrient levels in the soil and water availability are important. Cold temperatures usually cause dormancy decay (stratification) but, in some species, seeds need a warm period or even a heat shock to germinate. Light is very important for germination in many species (Borthwick et al. 1952). In some seeds, light acts as a promoter of dormancy (cereals and other large-seeded species), while in other seeds light acts as a promoter of germination (*Arabidopsis* and other small-seeded plants; Milberg et al. 2000). Another factor with a principal role in the seed dormancy release is time. The process usually known as after-ripening is a period of dry storage during which dormancy decreases. Temperature can influence after-ripening time in many seeds.

The balance between two hormones, gibberellins (GAs) and abscisic acid (ABA), is likely to play a key role in controlling germination and dormancy in seeds. GA has been described in the literature as a positive regulator of germination, promoting the growth potential of the embryo. In contrast, ABA synthesized by the embryo promotes dormancy (Karssen et al. 1983; Frey et al. 2004). ABA has also been found to be especially important in the endosperm repression of germination by blocking cell wall weakening in the micropylar endosperm, which precedes radicle emergence in germinating seeds (Müller et al. 2006). After-ripening has little effect on ABA content of dry *Arabidopsis* seeds. However, following imbibition, ABA content decreases rapidly by 50–70% in after-ripened seeds compared to dormant seeds. It is not yet clear where this decline in ABA occurs in after-ripened seeds, but evidence from gene knockout experiments indicates that the ABA 8′-hydroxylase, CYP707A2, plays a major role in the degradation of ABA in after-ripened seeds (Ali-Rachedi et al. 2004; Kushiro et al. 2004; Millar et al. 2006; Okamoto et al. 2006). So far, many genes have been found to be involved in GA or ABA metabolism or signalling in seeds. Some of these are controlled by environmental factors, and some have well-defined spatial and temporal expression patterns within the seed. These patterns highlight the complexity of the regulation of these two hormones, under the control of numerous factors which can act at different stages during seed development and in different tissues/organs, such as the endosperm or embryo. Mutagenesis and other approaches have also showed that transcription, translation and protein stability mechanisms are important for the proper dormancy control (Fig. 18.1b) and, recently, it has been shown that mutations affecting the chromatin structure and the epigenetic information can also affect the dormancy of seeds (Liu et al. 2007).

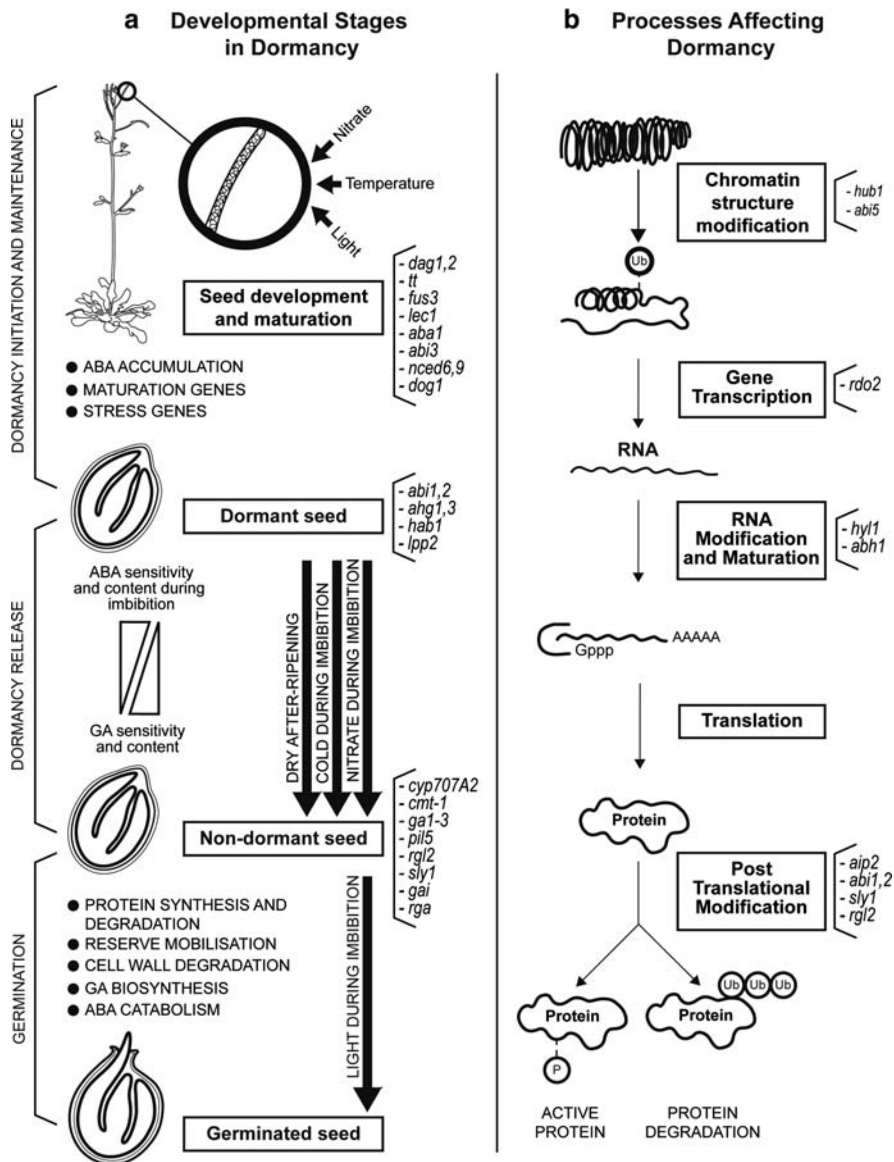

Fig. 18.1 The complexity of seed dormancy control. **a** Developmental stages in dormancy phases. Seed dormancy is initiated in the mother plant, during seed development and maturation. In this phase, environmental factors affect the concentration of ABA in the seed. ABA-, maturation- and stress-related genes are highly expressed. The result is a mature dormant seed with high ABA content and sensitivity. The transition between dormant and non-dormant phase is a gradual process in which ABA content and sensitivity decreases and GA content and sensitivity increases. These happen during after-ripening in a process which is not well understood, and is accelerated by cold and nitrate treatments. At some stage during after-ripening, the dormancy disappears and the seed is then receptive to signals like light which trigger the germination, involving protein

18.2 Approaches for Discovering Dormancy-Related Genes

Many different strategies have been used to dissect and modify the dormancy of seeds. Some of the approaches, as in other areas of plant biology, are based on reverse genetics, while others are based on forward genetics. The most common technique is mutagenesis (reviewed in Bentsink et al. 2007). Several dormancy mutants have been isolated, and some of the target genes have been cloned and their biological functions investigated. Quantitative trait loci (QTL) analysis is another technique for isolating genomic regions which contribute to grain dormancy. This approach has been useful in providing markers for breeding programs.

Full genome analyses of transcription can be accomplished (e.g. as in *Arabidopsis* and rice) with the sequencing of plant genomes and the development of high-throughput analyses platforms. Using bioinformatics tools, the researcher can compare expression profiles in wild-type and mutant seeds which have different dormancy states (reviewed in Holdsworth et al. 2008). Proteome analyses allow comparisons of protein expression profiles, but are limited to detection of more-abundant proteins. The metabolomic approach may also be useful. HPLC and GC-MS analyses permit the accurate quantification of changes in multiple hormones, for example, during dormancy development, maintenance and release.

In the following sections we will summarize results of different approaches to understand seed dormancy. In addition, we will discuss novel approaches which could be useful for identifying new dormancy genes and also strategies for modifying dormancy in cereals.

18.2.1 Mutagenesis

The classical reverse genetic strategy involves mutagenesis. Several mutant screenings have been done in *Arabidopsis*, and they have produced a large pool of mutants which display dormancy phenotypes (reviewed in Bentsink and Koorneef 2002; Bentsink et al. 2007). The study of these mutants has provided evidence about the role of different factors like GA, ABA, light, temperature, nitrate and the seed coat in dormancy establishment or release, and also about the role of processes like epigenetic regulation, protein degradation and RNA modification in dormancy control (Fig. 18.1). Most of the mutants with altered dormancy were found in screens which did not target dormancy, but dormancy phenotypes appeared as components of pleiotropic phenotypes. This indicates the complexity that exists

metabolism, reserve mobilization and cell wall degradation. **b** Transcriptional and posttranscriptional processes which affect seed dormancy. General processes affecting the gene expression through chromatin modification can affect dormancy. Downstream, RNA transcription, maturation and translation are also important in dormancy regulation. Finally, at the protein level there are many processes, like ubiquitination, which affect dormancy as well. Some *Arabidopsis* mutants affecting different processes are indicated as examples (see the text for details)

in dormancy control, and that many dormancy-related genes are involved in other processes. Most mutants have been found in *Arabidopsis*, but some have been isolated from barley, wheat, maize and rice. A common problem in these mutant screens is the difficulty in distinguishing between dormancy and germination, and between different types of dormancy (reviewed in Finch-Savage and Leubner-Metzger 2006). Very often, mutants with abnormal germination programs are considered as dormancy mutants, but this is not necessarily true. For example, phytochrome perception and signalling mutants have altered germination phenotypes, but this does not imply changes in dormancy initiation, maintenance and release.

Some of the first mutants showing dormancy phenotypes were related to defects in seed maturation. For example, the mutants *fusca 3* (*fus3*), *leafy cotyledon 1* (*lec1*) and *ABA-insensitive 3* (*abi3*) show a non-dormant phenotype, and all are affected in late maturation processes (Parcy et al. 1997), indicating that the dormancy has not developed correctly. This set of genes does not appear to be differentially regulated in dormant and non-dormant seeds (Baumbusch et al. 2004). Another group of mutations which exhibit reduced dormancy are those affecting the testa and endosperm structure. This is the case for *transparent testa* (*tt*) mutants (Debeaujon et al. 2000), in which the defective seed coat is not able to resist the embryo growth potential or to perceive correctly environmental factors like temperature (Tamura et al. 2006). It is clear from the study of these mutants that correct seed development is essential for dormancy initiation and maintenance. Maternal influences also have important roles in the establishment of dormancy. For example, *dof-affecting germination 1* (*dag1*) and *dag2* (Papi et al. 2000; Gualberti et al. 2002) mutants influence seed dormancy via a maternal effect (Fig. 18.1a).

The key role of ABA in dormancy is strongly supported by mutation studies. Mutant lines showing ABA-deficient or -insensitive phenotypes have been isolated and many of these show decreased seed dormancy. For example, the *ABA-deficient* (*aba*) and *ABA-insensitive* (*abi*) mutants belong to this group (Koornneef et al. 1982, 1984). Mutants that show ABA hypersensitivity or ABA accumulation and, as expected, produce seeds with increased dormancy. The *homology to ABI1* (*hab1*; Saez et al. 2004) mutant and the *ABA 8′-hydroxylase* mutants (Okamoto et al. 2006; Millar et al. 2006) respectively are examples. However, not all mutations in ABA-related genes show changes in dormancy which can be explained by redundancy with other genes or by these mutations having roles in other ABA-mediated processes. Many of these genes have specific temporal and spatial expression patterns, indicating that ABA may play different roles in different seed tissues (reviewed in Yamaguchi et al. 2007). A good example of this is the *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE* (*NCED*) gene family which encodes a key ABA biosynthetic enzyme (Tan et al. 2003). Of the five members in the family, only *NCED6* and *NCED9* are expressed highly in the seed, and only *NCED6* is present specifically in the endosperm, while *NCED9* is found in the embryo and endosperm (Lefebvre et al. 2006).

In addition to changes in ABA content in hydrated seeds, after-ripening also causes loss of ABA sensitivity in cereals (Walker-Simmons 1987; Benech-Arnold

et al. 1999; Corbineau et al. 2000) and, in *Arabidopsis*, the *abi1*, *abi2* and *abi3* mutants have reduced dormancy. A key component of the ABA signalling pathway in dormancy is a protein phosphatase 2C (PP2C) clade. This clade has nine members which are involved in ABA signalling in *Arabidopsis* (Nishimura et al. 2007). Based on expression analysis of PP2C genes, it has been shown that *ABA-HYPERSENSITIVE GERMINATION 1* (*AHG1*) and *AHG3* have strong expression in seeds. Compared with the wild type, the *ahg1-1* and *ahg3-1* mutants show a deeper dormancy, and double mutant analyses show that these two genes act upstream of *ABI3* and *ABI5*, which are key transcription factors in ABA signalling. GUS-promoter fusions show that these two genes are expressed in both embryo and endosperm tissues. The expression of these genes in the endosperm is very interesting because a number of studies have shown the importance of this tissue in maintenance of dormancy (Bethke et al. 2007; Nishimura et al. 2007). The pattern of gene expression in the endosperm is clearly different from the embryonic pattern, being similar to the profile found in dry seeds and dormant imbibed seeds, and different to the profile found in non-dormant imbibed seeds (Holdsworth et al. 2008). *ABI3* and *ABI5* are expressed in the embryo and endosperm (*ABI5* in the micropylar endosperm cap; Penfield et al. 2006). Gene expression analysis has demonstrated that the expression of *ABI5* is much more strongly induced by ABA in the *ahg1-1* and *ahg3-1* mutants, indicating that these two genes could be very important in the ABA signalling pathway in the endosperm, and that they could affect the level of dormancy in seeds (Nishimura et al. 2007).

Mutations in genes related to GA metabolism and signalling have been reported to alter dormancy and germination. The phenotype of these mutants is usually pleiotropic, making it difficult to identify a role for GA in seed dormancy. Such is the case for *GA requiring 1-3* (*ga1-3*; Sun et al. 1992) or *sleepy 1* (*sly1*; Steber et al. 1998). The former cannot germinate unless GA is added to the medium, while the latter produces seeds with very poor viability. DELLA proteins are important in the GA signalling pathway (Dill et al. 2001). These proteins are negative regulators of GA signalling, and are degraded via 26S proteosome in the presence of GA and in response to light (Fu et al. 2002; Itoh et al. 2003). One of the DELLA proteins, RGA-LIKE 2 (RGL2), has been shown to have a seed-specific role, mediating the repression of GA signalling and germination (Lee et al. 2002). The *rgl2* mutants have reduced dormancy and could be very useful for elucidating the GA-related mechanism involved in seed dormancy. The *COMATOSE* (*CMT*) gene is a promoter of germination and has been implicated in GA signalling (Russell et al. 2000). The *cmt-1* mutant fails to germinate and has been found to be epistatic to the *rgl2-1* mutant (Carrera et al. 2007). Interestingly, *cmt-1* and wild-type seeds have similar transcriptome changes during after-ripening (Carrera et al. 2007), suggesting that GA has two roles, one in germination and the other in dormancy.

Light perception is an important factor controlling dormancy and germination in plants (Borthwick et al. 1952). Red and far-red wavelengths are active components of the light spectrum regulating germination in *Arabidopsis* (Shinomura et al. 1994), and this response is mediated mainly by phytochrome B (Shinomura et al. 1996). The PHYB signalling pathway regulates germination by modifying the

expression of GA and ABA metabolism genes (Yamaguchi et al. 1998; Oh et al. 2006; Seo et al. 2006). Red light promotion of germination in *Arabidopsis* acts in part through induction of the GA biosynthetic genes *GA3ox1*, *GA3ox2*, and repression of the ABA biosynthetic genes *ABA1*, *NCED6* and *NCED9*. Red light has the opposite effect on GA and ABA catabolism, increasing the expression of the ABA catabolic gene *CYP707A2* and reducing the expression of the GA catabolic gene *GA2ox* (Oh et al. 2007). These light responses are mediated by PHYTOCHROME INTERACTING FACTOR 3-LIKE 5 (PIL5) protein, which is targeted to the proteosome and degraded in response to light. PIL5 regulates directly the transcription of two DELLA proteins, GA INSENSITIVE (GAI) and REPRESSOR OF GA1-3 (RGA), which regulate GA and ABA metabolic genes indirectly (Oh et al. 2007). Despite the importance of these interactions for understanding the mechanism which triggers germination, it is not clear if a connection with dormancy exists, and how after-ripening or other dormancy release factors modify this light signalling cascade. In contrast to *Arabidopsis* seeds, red and far-red light appear to have no effect on germination or ABA content in barley grains. Recent work has shown that blue light promotes dormancy in freshly harvested barley grains. Gene expression studies and hormone analyses indicate that the blue light response is caused by induction of *HvNCED1* (Gubler et al. 2008).

Mutants affecting the response to, or the synthesis of other hormones, like ethylene or brassinosteroids, also show altered dormancy phenotypes (reviewed in Finkelstein et al. 2008). It is difficult to say if this effect on dormancy is direct or indirect, due to complex interactions between ethylene, brassinosteroid and ABA response pathways (Chiwocha et al. 2005).

Only one group of mutants, *reduced dormancy 1-4* (*rdo1-4*), was isolated specifically through a dormancy screen (Leon-Kloosterziel et al. 1996; Peeters et al. 2002), and even these mutants display pleiotropic effects like changes in stem and silique length or in leaf colour. *RDO4* has recently been cloned and renamed *HISTONE MONOUBIQUITINATION 1* (*HUB1*), and has a role in the monoubiquitination of histones (Liu et al. 2007). *RDO2* has been also cloned and encodes a transcription elongation factor (Geyer et al. 2007). Both mutants suggest that general mechanisms controlling gene transcription and epigenetic information may be important in dormancy regulation. Also, other general mechanisms involving posttranscriptional RNA processing, maturation and metabolism appear to be related to dormancy, because many mutants affected in these RNA processes, like *ABA hypersensitive 1* (*abh1*; Hugouvieux et al. 2001) and *hyponastic leaves 1* (*hyl1*; Lu and Fedoroff 2000), have altered dormancy.

Along with transcriptional and posttranscriptional regulation, mechanisms targeting protein stability, like the ubiquitination pathway, are also important in dormancy control (Fig. 18.1b). This pathway targets proteins for degradation by the 26S proteosome (reviewed in Smalle and Vierstra 2004), and has been shown to be crucial for GA and ABA signalling. DELLA, ABI3 and ABI5 proteins are all regulated by ubiquitin-mediated proteolysis (Lopez-Molina et al. 2003; Zhang et al. 2005). It is therefore to be expected that mutation in this pathway would affect

dormancy and germination. For example, *SLY1* (McGinnis et al. 2003) is an F-box protein which acts in the SCF E3 ubiquitin ligase complex, targeting DELLA proteins for degradation (Fu et al. 2004), and the *sly1* mutant has a constitutive block in GA response. Another example is ABI3-INTERACTING PROTEIN 2 (AIP2), a RING finger E3 ligase which targets *ABI3* for degradation (Zhang et al. 2005). The *aip2-1* mutant shows ABA hypersensitivity. A common mechanism linking early GA and ABA responses in germination, and also ubiquitination, has been described (Zentella et al. 2007). In this work, 14 DELLA target genes were found by means of microarray analysis and chromatin inmunoprecipitation. The expression of these genes was repressed by GA and promoted by DELLA. One of these genes is *XERICO* (Ko et al. 2006), a small RING finger putative E3 ligase which promotes the accumulation of ABA. The study of the interactions between *XERICO* or other RING finger E3 ligases and DELLA proteins, especially RGL2, could be very important for understanding the mechanism controlling ABA and GA common regulatory elements.

Several other dormancy mutants have been described for other species. The better known are the viviparous mutants from maize. Some of these, like *VIVIPAROUS 1* (*VP1*) and *VP14* genes, are related to ABA, encoding the orthologous forms of the *Arabidopsis ABI3* and *NCED* respectively (McCarty 1995; Schwartz et al. 1997). In rice, only one mutant gene has been cloned and, again, it is related to ABA (Agrawal et al. 2001). In barley, one mutant with reduced dormancy has been isolated (Romagosa et al. 2001) but, to date, the gene has not been cloned.

18.2.2 QTL Analysis

Another genetic tool which has been extensively used to probe dormancy is QTL analysis. This approach has been used for *Arabidopsis* to identify genes responsible for differences in dormancy between different ecotypes. *Arabidopsis* recombinant inbred lines have been constructed using ecotypes with different dormancy behaviours, and then used in QTL analyses (van der Schaar et al. 1997; Alonso-Blanco et al. 2003; Clerkx 2004). Similar regions have been identified using different mapping populations, indicating that those loci are conserved in a number of ecotypes, supporting their importance in dormancy. The next step for the cloning of the gene(s) responsible for the QTL is the construction of near-isogenic lines. This has been done for the Cvi/L*er* population, allowing the cloning of the first QTL involved in dormancy, namely *DELAY OF GERMIANTION 1* (*DOG1*; Bentsink et al. 2006). The function of *DOG1* is still unknown but it appears to be related to ABA metabolism. It is expected that genes responsible for other dormancy QTLs will soon be identified.

QTL studies have also been used in cereals, especially in rice, wheat and barley (Ullrich et al. 1993; Lin et al. 1998; Kato et al. 2001). In barley, for example, up to 27 dormancy QTLs have been identified (Ullrich et al. 1993). Once a major

QTL has been identified, molecular markers can be developed to assist the introduction of the QTL into breeding programs. For example, a major dormancy QTL in wheat, the 4AL QTL (Mares et al. 2005), has been identified in three bread wheat genotypes with different origins. This QTL could be related to sensitivity of the embryo to ABA (Noda et al. 2002). Molecular markers which are tightly linked to the QTL have been developed and are being used in breeding programs (Zhang et al. 2008). Very recently, a new QTL analysis for pre-harvest sprouting has been done in wheat (Imtiaz et al. 2008). In this work, a new mapping population was constructed using the diploid wild dormant wheat *Aegilops tauschii* and the tetraploid low dormant variety *Triticum turgidum*. Nine major QTLs were found, proving the importance of the wild germoplasm for addressing the pre-harvest sprouting in domesticated varieties. Despite the importance of the QTLs in cereal breeding programs, genes responsible for the trait have not been cloned.

18.2.3 Proteomics

Changes which occur in the proteome associated with different dormancy states have been analyzed (reviewed in Holdsworth et al. 2008). The experiments (Rajjou et al. 2004) using α-amanitin and cycloheximide indicate that translation appears to be essential for germination, while transcription does not. This underlines the importance of stored mRNA for seed germination. Another study (Gallardo et al. 2002) compared the proteomes of L*er* seeds and GA-deficient mutants, after 24 h of imbibition. This experiment showed interesting results regarding the proteins affected by GA during germination, but did not investigate differences between dormancy states.

A comparison of the proteomes of dormant and non-dormant Cvi seeds of Arabidopsis has shown differential accumulation of 32 proteins which are related mainly to metabolic functions during after-ripening in dry seeds (Chibani et al. 2006). In this study, it was also demonstrated that the application of external ABA to non-dormant seeds blocks germination, but does not mimic the proteome state of the dormant seeds. This shows that once dormancy is lost, external ABA application probably activates stress signals, thereby modifying the germination potential through a different pathway to dormancy. Proteome analysis has provided very useful results in *Arabidopsis*, where many proteins can be easily identified due to genome sequence availability (Fu et al. 2005). It would be of interest to compare the proteome from different tissues in dormant and non-dormant seeds, and to examine changes due to mutations which affect dormancy, such as *dog1*.

In cereals, proteomic approaches have so far focused on germination, rather than dormancy. The results from rice are very similar to those found in *Arabidopsis*: storage proteins, and proteins related to seed maturation and stress are down-regulated during germination, while proteins related to reserve mobilization are up-regulated (Yang et al. 2007).

18.2.4 Metabolomics

Another approach which can be used for unmasking the processes underlying dormancy maintenance and release is metabolomics. The profiles of different hormones and hormone metabolites, during imbibition of dormant and non-dormant seeds, provide valuable insights into dormancy changes or dormancy states. Metabolomics can be applied to help understand the complex crosstalk between hormones in mutant phenotypes (Chiwocha et al. 2003). For example, ethylene mutants, which have altered dormancy phenotypes, showed altered levels of ABA, GA and other hormones (Chiwocha et al. 2005). It is not clear whether the effect of ethylene on dormancy is direct or indirect via changes in GA and ABA.

Other compounds, like nitric oxide and other reactive oxygen species, can be very important for defining different seed dormancy states, and have been found to be important in dormancy release (Bethke et al. 2004). The analysis of mobilization of lipids and other reserve molecules can be also an indicator of dormancy release, and this can be done in a tissue-dependent manner (reviewed in Penfield et al. 2007). New techniques are appearing which allow the quantification of molecules in vivo. For example, a quantitative imaging system has been developed for measuring lipid content in living cells, and has been used to track changes occurring during barley and soybean grain development (Neuberger et al. 2008).

A carefully analysis of the metabolome in different tissues and developmental stages, and after different imbibition times, could provide specific signatures for each situation, and these signatures could be very useful in identifying important processes and the genes responsible for those processes.

18.2.5 Gene Expression Analysis

Two recent microarray studies have analyzed the transcriptomes which are associated with different dormancy states in *Arabidopsis* Cvi seeds (reviewed in Holdsworth et al. 2008). The first of these showed that dormant and non-dormant seeds have different gene expression profiles (Cadman et al. 2006). Four hundred and forty-two genes were found to be up-regulated in dormant seed, compared with non-dormant seeds. In this group, ABA-related, seed maturation-related and stress-related genes were abundant. Seven hundred and seventy-nine genes were up-regulated in non-dormant seeds and many were related to protein synthesis and protein degradation. Also, reserve degradation and cell wall modification genes were overrepresented. It is also clear, from quantitative real-time PCR studies, that genes involved in the synthesis or catabolism of ABA and GA play a major role controlling dormancy (Millar et al. 2006; Gubler et al. 2008). Genes involved in ABA synthesis and GA degradation are more highly expressed in dormant seeds, and genes involved in ABA degradation and GA synthesis are more highly expressed in non-dormant seeds. A correlation between increased expression of

ABA catabolism genes and GA synthesis genes following after-ripening has also been observed in barley (Chono et al. 2006; Millar et al. 2006; Gubler et al. 2008). Similarities have been observed in transcriptome profiles of strongly dormant and weakly dormant ecotypes, indicating that common mechanisms control both states (Carrera et al. 2007). However, differences also exist and we cannot rule out the possibility that different mechanisms establish the deep dormancy state in Cvi and the weak dormancy in L*er* or Col.

The second microarray study analyzes how different environmental factors, like after-ripening, low temperature, nitrate and light, affect gene expression in dormant and non-dormant seeds (Finch-Savage et al. 2007). The results indicated that apart from after-ripening, these factors are dependent variables acting in similar ways, and their effects depend on the after-ripening time. The results also showed that the dry dormant and the dry non-dormant states are very similar, and that it is during imbibition that differences appear. It is clear again that environmental factors modify the ABA/GA balance through gradual quantitative changes which occur during after-ripening. In this study, a group of 30 genes appear to be related specifically with dormancy maintenance and release, showing a decrease in expression during after-ripening. The fact that *DOG1* appears in this group is strong support for their role in dormancy-related processes. Among the other genes, it is important to mention two PP2C phosphatases which could be involved in ABA signalling, and two ethylene response factors, which could indicate an important role for this hormone in dormancy release.

Analysis of the after-ripening process is complicated due to the difficulty in separating the dormancy and germination programs. A recent paper addresses this problem using the classical *aba1-1* (Koornneef et al. 1982) and *abi1-1* (Koornneef et al. 1984) mutants, which cannot synthesize or perceive ABA respectively (Carrera et al. 2008). These mutants do not show seed dormancy, indicating that changes in the transcriptome during after-ripening are independent of the dormancy states. Two groups of ABA-related genes appear to be positively or negatively regulated with after-ripening. In the first group the gene *LIPID PHOSPHATE PHOSPHATASE 2* (*LPP2*; Katagiri et al. 2005), which produces ABA hypersensitivity when mutated, may explain a decrease in ABA sensitivity in after-ripened seeds. In the second group, *ABA1* (Marin et al. 1996), the first enzyme in ABA biosynthesis, may explain a higher ABA content in dormant seeds (Carrera et al. 2008).

Gene expression profiles have also been examined in wild-type Col and in mutant *abi4-11* and *abi5-7* seeds, at the dry stage and 6 h after imbibition (Nakabayashi et al. 2005). Interestingly, the results show that the differentially expressed genes after imbibition are clustered, and that the genes of which the expressions are affected by the *abi5-7* mutation are also localized in clusters which are susceptible to DNA methylation. These results indicate that the expression profile during seed development could be controlled by epigenetic mechanisms like methylation and, together with the dormancy phenotype of *hub1* (Liu et al. 2007), strongly support a role for chromatin modification in seed development and dormancy maintenance.

Comparison of the transcriptome during maturation, desiccation and germination in barley grains, using the Affymetrix barley1 chip (Close et al. 2004), has been published recently (Sreenivasulu et al. 2008). This work analyzes changes in both the endosperm and the embryo. The authors found that more than 12,000 different transcripts were stored in dry grains and that, at the early stages of germination, many genes involved in reserve mobilization showed changes in expression levels within 24 h of the commencement of imbibition. Many transcripts related to ABA and GA metabolism appear specifically in the aleurone, indicating a major role for this tissue during seed maturation and germination. Also, many transcripts related to ethylene and auxin appear to be induced during germination, indicating that a complex relationship exists between hormones.

In barley, other approaches for finding genes related to dormancy have been used. Using cDNA-AFLP, a group of transcripts has been found to be differentially expressed by dormant and non-dormant embryos, and some of these are related to epigenetics and ABA signalling (Leymarie et al. 2007). New high-throughput platforms are available which facilitate the study of the expression of all the transcription factors from *Arabidopsis* and rice, using real-time RT-PCR. This approach has been employed in *Arabidopsis* to identify new genes involved in root and shoot development (Czechowski et al. 2004), and in phosphate deprivation (Morcuende et al. 2007). In rice, it has been used to study 2,500 transcription factors (Caldana et al. 2007). It has also being used in C24 seeds of *Arabidopsis* for analyzing the dormant and non-dormant states (Barrero et al. in preparation). Of 2,236 transcription factors, 39 were differentially expressed between dormant and non-dormant seeds during imbibition. After analyzing T-DNA insertions lines for these genes, two displayed an increased or reduced dormancy level compared with the wild-type Col. This strategy allows the study of dormancy-related transcription factors which are often undetectable in microarray approaches because of their low expression values. This forward genetic tool can be combined with reverse approaches. Once a candidate gene has been selected, the effect of its mutation or overexpression must be determined at the phenotypic level. The large T-DNA mutant collections available in *Arabidopsis* facilitate phenotype identification but, in crop species, this can be more complicated because of the smaller size of mutant collections and because their polyploidy can result in gene redundancy.

18.3 Strategies for Modifying Dormancy in Cereals

The use of *Arabidopsis* as a model plant to identify molecular mechanisms of dormancy provides an excellent starting point for cereal research (Wilkinson et al. 2002). After isolating candidate genes, the crop orthologues must be functionally tested. Different strategies like overexpression, mutagenesis, TILLING or the designing of synthetic microRNAs can be used for up- and down-regulation of their expression. A similar strategy has been followed for manipulating the expression of *ABI3* and *FUS3* orthologues in wheat (Wilkinson et al. 2002).

A recent example of this combined approach is the work done with the ABA 8′-hydroxylase enzyme in *Arabidopsis* and barley. ABA catabolism has been found to be important in the control of dormancy and germination in *Arabidopsis*. The *CYP707A1-4* genes of *Arabidopsis* encode 8′-hydroxylases which convert ABA to phaseic acid (Kushiro et al. 2004). *CYP707A1* and *CYP707A*2 show specific expression patterns during seed maturation. CYP707A2 knockout results in higher ABA content and increased dormancy in seeds, while overexpression has the opposite effect (Millar et al. 2006; Okamoto et al. 2006). *CYP707A2* is highly expressed in after-ripened seeds and has been proposed to be responsible for the lower ABA content in imbibed after-ripened seeds, compared to dormant *Arabidopsis* seeds (Millar et al. 2006). Its orthologue in barley, *HvABA8′OH1*, is also expressed more strongly in embryos from non-dormant grains. Barley is a diploid cereal which can be easily transformed, making it a good experimental plant for testing gene function—in this case, whether down-regulation of *HvABA8′OH1* expression results in increased dormancy, as found in *Arabidopsis*. In such a study (Gubler et al. 2008), RNA interference (RNAi) constructs were prepared for targeting the barley *HvABA8′OH1*, and transformed into Golden Promise barley. Transgenic plants expressing the RNAi gene against *HvABA8′OH1* had higher ABA content in the embryo and, consequently, a deeper dormancy than was the case for non-transgenic lines. Following the success of the RNAi targeting of the *HvABA8′OH1* gene, it is expected that RNAi and associated synthetic micro-RNA silencing technologies will be successfully used for other dormancy-related genes in barley.

A novel gene silencing technique called chimeric repressor gene-silencing technology (CRES-T) can be used for determining the effect of silencing a transcription factor in a dominant way. In this technique, a chimeric repressor is produced by fusion of a transcription factor to the plant-specific ERF-associated amphiphilic repression (EAR)-like motif repression domain (Hiratsu et al. 2003). CRES-T has been used recently for silencing transcription factors involved in secondary wall formation, morphology of lateral organs and pollen development (Ito et al. 2007; Koyama et al. 2007; Mitsuda et al. 2007), and its effectiveness has been tested in several plant species.

For manipulating gene expression in plants which will be used in breeding programs, transgenic approaches have strict regulatory requirements. Large collections of mutated and wild-type germplasm are available in rice, barley and wheat, and it is possible to use PCR-based molecular techniques to find lines with mutations in any gene (e.g. Gubler et al. 2008). A new approach based on targeting induced local lesions in genomes (TILLING; Colbert et al. 2001) can be used to accelerate the identification of natural mutant varieties. Ecotilling is a high-throughput technique which allows the isolation of different kinds of polymorphisms in natural populations (Comai et al. 2004). It is based on the formation of heteroduplex structures in pooled DNA samples, coming from different genotypes. After amplifying a fragment of interest using PCR, a heteroduplex structure can be identified by *Cel*I restriction enzyme digestion (Oleykowski et al. 1998). The Ecotilling protocol can be adapted for many species (Till et al. 2006), and could

be very useful in comparative genetics and for applying the results found in *Arabidopsis* to other species.

18.4 Conclusions and Perspectives

Research on seed dormancy using *Arabidopsis* has produce promising outcomes which can be applied in cereals because many similarities exist between the dormancy behaviour in these two types of species. The responses to cold, after-ripening and nitrate are very similar in *Arabidopsis* seeds and barley grains. Also, the effects of changes in ABA and GA contents and sensitivities to these hormones appear to be similar and critical in all plants studied so far.

Nevertheless, some important differences in seed structure exist between *Arabidopsis* and cereals which may impact on dormancy mechanisms. In *Arabidopsis*, the root tip abuts the micropylar endosperm, while in cereals the primary roots are surrounded by the coleorhiza. Structural and physiological differences between micropylar endosperm and coleorhiza may be important issues for blocking root elongation in dormant seeds. Such differences may limit the use of *Arabidopsis* as a model for cereal dormancy. The use of a monocotyledon model plant like rice may overcome this problem but, because of the large genome size of rice and because many rice varieties have low dormancy, its utility for studying dormancy mechanisms may be limited. The use of a new model plant for monocotyledons, *Brachypodium distachyon*, is expected to produce new and promising results. It is phylogenetically close to important crop plants like wheat and barley, it exhibits agricultural traits similar to those found in crops, and it has a short life cycle (Ozdemir et al. 2008). Many diploid accessions are already available, and the genetics tools for working with *Brachypodium* are increasing rapidly. The fact that it is an undomesticated wild grass presents opportunities for discovery of new genes related to seed dormancy.

Note: a new transcriptomic study has been published while this book chapter was in end production. The authors used a tissue-specific microarray approach for comparing dormant and after-ripened barley grains during early imbibition (Barrero et al. 2009). Based on the results, it is proposed that the coleorhiza plays a major role in dormancy in cereals, acting as a barrier to root emergence in dormant grains, and realising this block during after-ripening.

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Chapter 19
Seed Germination

R.C. Martin, W.E. Pluskota, and H. Nonogaki

19.1 Introduction

Seed germination is the emergence of a new plant from seed. A quiescent embryo contained in a dry seed becomes active after hydration (imbibition), although dormant seeds still do not germinate after full hydration under optimal conditions. In a narrow scientific definition, seed germination is complete when the radicle penetrates through the covering tissues such as the testa (seed coat) and endosperm. In a practical sense, the initial growth of germinated seeds through the establishment of healthy seedlings can also be referred to as seed germination. While seedling growth can be monitored visually, it is difficult to observe the changes in germinating seeds when the embryo is still enclosed by the testa and endosperm. Integration of molecular genetics, hormone biology, and bioinformatics has made it possible to dissect the complicated mechanisms of seed germination. Recent progress in seed germination biology is summarized in this chapter.

19.2 Seed Structure and Germination

Understanding the structure of seeds, and the genetic origin of seed tissues is essential to analyze the mechanisms of seed germination. Elucidating the mechanisms of seed germination is important to modify seed germination performance

R.C. Martin
USDA-ARS, National Forage Seed Production Research Center, Corvallis, OR 97331, USA
W.E. Pluskota
Department of Plant Physiology and Biotechnology, University of Warmia and Mazury, Oczapowski 1A 10-718, Olsztyn, Poland
H. Nonogaki
Department of Horticulture, Oregon State University, Corvallis, OR 97331, USA
e-mail: hiro.nonogaki@oregonstate.edu

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through breeding programs, and to develop biotechnology for seed enhancement. The major seed structures are the embryo, which becomes a plant, the endosperm, which provides nutrition for a germinated seedling, and the testa, which protects the embryo and endosperm from harsh environments. It is important to note that these three tissues have distinct genotypes. The embryo is a diploid (2n) tissue originating from the fusion of a haploid (n) male gamete from pollen, and a haploid egg cell in the ovule. The endosperm is a triploid (3n) tissue resulting from the fusion of the other haploid male gamete and the central nuclei in the maternal tissue. By contrast, the testa is independent of fertilization and derived from integuments, purely maternal tissues. As each seed component affects seed dormancy and germination, understanding the origin of each tissue is critical for genetic modification of seed traits.

19.2.1 Testa and Pericarp

The testa of seeds in many species contains dark (brown to black) pigments. In *Arabidopsis thaliana* (called Arabidopsis hereafter) seeds, pigmentation of the testa is observed at late stages of seed development. However, actual synthesis of the pigments, which are called proanthocyanidins (PA) or condensed tannins, starts during early stages of embryo development (1–2 days after fertilization; Debeaujon et al. 2007). These flavonoids initially accumulate as colorless compounds in vacuoles of the endothelium, the innermost cell layer of integuments, are oxidized during seed desiccation, and confer the brown color of mature seeds (Debeaujon et al. 2007). The pigments contribute to the chemical and physical properties of testa, and impose seed dormancy. When embryos are excised from dormant seeds, they are capable of growing. This indicates that Arabidopsis seed dormancy is a coat-imposed dormancy. The role of testa pigmentation in seed dormancy is obvious, because seeds of testa pigmentation mutants called *transparent testa* (*tt*), in which the testa exhibits white to pale yellow color, lack dormancy (Debeaujon and Koornneef 2000). Many *TT* genes encode enzymes in the flavonoid biosynthesis pathway (Feinbaum and Ausubel 1988; Shirley et al. 1992), while others encode transcription factors acting upstream of the flavonoid biosynthesis enzymes (Nesi et al. 2000, 2001, 2002), or the membrane transporter proteins responsible for PA accumulation in vacuoles (Debeaujon et al. 2001; Baxter et al. 2005). Genetics of *tt* mutation provides a good example of the importance of understanding tissue origin. Because the testa is derived from the integuments, *tt* phenotypes are inherited maternally—that is, when seeds are produced on *tt* plants, all progeny seeds lack pigments and dormancy, regardless of the genetic information of the embryo. This maternal inheritance is important when this type of information is applied to breeding programs, and genetic modification through biotechnology. Isolation and intensive characterization of *tt* mutants in the last decade greatly advanced our knowledge of coat-imposed dormancy in seed biology. The information is now available to be translated into crop species.

Another important aspect of testa properties is the mucilage deposited on the surface of testa. The testa color of *myb61* mutant Arabidopsis seeds is normal. However, these seeds produce a reduced amount of rhamnose and galacturonic acid, important components of rhamnogalacturonan (RG)-containing pectins, and are deficient in seed mucilage production (Penfield et al. 2001). Germination of *myb61* seeds is reduced in an osmoticum compared to wild-type seeds, suggesting that seed mucilage may play a role in promoting imbibition and/or the retention of water around seeds (Penfield et al. 2001). Likewise, a mutation in *MUM4* (*MUCILAGE-MODIFIED4*) that encodes an enzyme necessary for galacturonan synthesis also causes a reduction in seed mucilage accumulation. While the above studies indicate that a certain amount of mucilage is important to make the natural hydrogel surrounding seeds, recent studies demonstrated that qualitative changes in seed mucilage could cause reduced hydrogel phenotypes. Seeds of the *mum2* mutant produce a normal amount of polysaccharides. However, the mucilage still fails to expand upon hydration in these mutant seeds. *MUM2* encodes a β-galactosidase that may be involved in the synthesis of a more branched rhamnogalacturonan I than that of wild type (Dean et al. 2007; Macquet et al. 2007). These results suggest that specific chemical properties of mucilage are necessary for proper expansion, and the formation of natural hydrogel during germination. These compounds, or knowledge about these compounds, could be applied to seed treatment technologies such as film coating and pelleting.

There are many plant species in which the dispersal units are not true seeds such as those of Compositae (e.g., lettuce and sunflower), Umbelliferae (e.g., carrot and parsley), Chenopodiaceae (e.g., spinach and beet), and Graminaceae (e.g., rice and wheat) species. In these species, dispersal units are fruits, and therefore the outermost layer of the dispersal units is not testa, but pericarp. The properties of the pericarp are also critical for seed dormancy and germination. In sugar beet (*Beta vulgaris*) seeds, germination is restricted by the pericarp, which retains abscisic acid (ABA), a germination-inhibiting plant hormone (Hermann et al. 2007; see below for more about ABA). The pericarp of *Zinnia elegans* also inhibits seed germination. Removal of the pericarp, extraction of ethanol-soluble inhibitors from the pericap, or oxidation of the inhibitor by hydrogen peroxide promotes seed germination in this species (Ogawa and Iwabuchi 2001). Thus, the outermost layer of the dispersal unit, testa or pericarp, contribute to the control of seed germination.

19.2.2 Endosperm

In endospermic seeds, which include many vegetable and flower seeds, the embryo is surrounded by the endosperm. The endosperm provides nutrition during seedling establishment through reserve mobilization. However, the endosperm can also exert negative effects on radicle emergence. The mechanical resistance of the endosperm can be an obstacle to radicle emergence. This has been well characterized in lettuce

(Halmer et al. 1975), pepper (Watkins et al. 1985), and tomato (Groot and Karssen 1987; Groot et al. 1988).

In tomato seeds, the micropylar region of the endosperm that is adjacent to the radicle tip of the embryo (called 'endosperm cap') provides mechanical resistance, and acts negatively on seed germination (Groot and Karssen 1987; Groot et al. 1988). Measurements of the force required to puncture the endosperm cap (puncture force) by a texture analyzer clearly indicated that this tissue is weakened before radicle emergence (Groot and Karssen 1987; Chen and Bradford 2000), supporting the idea that endosperm weakening is necessary for the induction of germination in tomato seeds. The rigidity of the endosperm cap is due mainly to the thick cell walls. Multiple cell wall modifying proteins, such as endo-β-mannanases (Groot et al. 1988; Nonogaki et al. 1992), xyloglucan endotransglycosylase/hydrolase (XTHs; Chen et al. 2002), and expansins (EXPs; Chen and Bradford 2000; Chen et al. 2001), are thought to be involved in this weakening process.

Arabidopsis seeds have a single cell layer of endosperm, which is sometimes referred to as the aleurone. This layer is not exactly the same as the aleurone layer in cereal seeds (caryopses), although the appearance of Arabidopsis endosperm cells is very similar to that of cereal aleurone cells. In cereal seeds, the endosperm is highly differentiated into two distinct portions, the starchy endosperm, generally non-living tissue at maturity, and the aleurone layer, living tissue capable of secreting enzymes to digest the starchy endosperm. However, Arabidopsis seeds have only a single cell-layer living endosperm. The major site of seed reserves in Arabidopsis seeds is the cotyledons, which are living tissues. Therefore, Arabidopsis endosperm is slightly different from cereal aleurone in terms of function. The Arabidopsis endosperm appears to negatively affect radicle emergence (Liu et al. 2005a). While the contribution of testa properties to seed dormancy is apparent, the dormant status of a seed still persists even after the testa is removed if the embryo remains in the endosperm layer (Bethke et al. 2007). Therefore, the endosperm is also a critical factor controlling Arabidopsis seed germination. It is not clear whether the micropylar region of the Arabidopsis endosperm provides substantial mechanical resistance. However, there are many occasions where testa rupture occurs, but the radicle tip cannot penetrate the endosperm layer (Liu et al. 2005b; Muller et al. 2006; Oh et al. 2007) which supports the idea of the suppression of germination by the endosperm layer.

19.2.3 Embryo

The details of embryogenesis are described in Chapter 17, and therefore are not covered in this chapter. However, since germination capacity of seeds occurs during embryo development, the developmental changes associated with seed germination are discussed. In Arabidopsis, the embryo goes through multiple developmental stages that are defined morphologically as globular, heart, torpedo,

walking-stick, and mature stages. The major events during early stages of embryogenesis are histodifferentiation. It is the later stages of embryogenesis that play a more important role in seed maturation. Germinability is not observed until the embryo reaches mature morphology in Arabidopsis (Raz et al. 2001). While morphological maturation is essential for the development of germination potential in Arabidopsis, not all mature embryos exhibit full germination potential. Morphologically mature and visually indistinguishable embryos isolated from different positions of developing siliques exhibit different germination potential. Relatively young mature embryos do not show full germination potential, while older ones germinate completely (J.R. Kristof and H. Nonogaki, unpublished data). These results suggest that physiological maturation of embryos is still required after they reach morphological maturity. Mature embryos generally go through desiccation tolerance, except for recalcitrant seeds that are not resistant to dehydration.

When mature seeds are imbibed in water, embryo growth potential is gradually generated, which eventually overcomes the restriction from covering tissues discussed above. Modification of cell walls within the embryo probably also plays an important role. In tomato seeds, *LeEXPA8* (an expansin gene) is expressed specifically in the elongation zone of the embryo (Chen et al. 2001). This gene may be associated with cell wall loosening and cell elongation, which provides the driving force for radicle protrusion. Likewise, multiple expansin genes (*AtEXP1*, *AtEXP2*, *AtEXP3*, *AtEXP6*, *AtEXP8*, and *AtEXP15*) are induced during Arabidopsis seed germination. In addition, *AtXTH* (xyloglucan endotransglycosylase/hydrolase), another gene associated with cell wall modification, is expressed exclusively in the embryonic axis (Ogawa et al. 2003). These findings suggest that cell wall loosening in the embryo is necessary for the generation of embryo growth potential.

Regulatory proteins that may be associated with the events upstream of cell wall modification in the embryo have been characterized. Enhancer-trap screening of seed germination-associated genes identified the *BME3* gene encoding a zinc finger transcription factor. The *BME3* transcript accumulates in seeds during cold stratification. The *bme3* knockout seeds exhibited testa rupture, but the embryo failed to penetrate the endosperm layer, suggesting that this gene may be important for the increase in embryo growth potential (Liu et al. 2005b). Another gene involved in the activation of embryo growth potential is *AtTCP14*, a member of the TCP protein family (TB1, CYC, and PCF; Tatematsu et al. 2008). This gene was originally discovered during a search for *cis* regulatory elements (Up elements), usually found in the promoter regions of genes that are upregulated specifically during Arabidopsis seed germination. AtTCP14 belongs to a family of bHLH-type transcription factors, and targets the site II motif that is nearly identical to the germination-specific Up1 *cis* element. *AtTCP14* is expressed mainly in the vascular tissue of the hypocotyl. Embryos in *attcp14* mutant seeds were hypersensitive to exogenously applied ABA and paclobutrazol (PAC), an inhibitor of gibberellin (GA) biosynthesis. Since ABA and GA are known to act negatively and positively on embryo growth, respectively, these results suggest that *AtTCP14* regulates the activation of embryo growth potential in Arabidopsis seeds (Tatematsu et al. 2008).

19.3 Hormonal Regulation of Germination

Many of the changes in the embryo and endosperm during seed maturation and imbibition described above are controlled by plant hormones such as ABA and GA. Changes in hormone concentrations in seeds play a critical role in the suppression and promotion of germination programs. Vivipary, precocious germination from seeds that are still developing on the maternal plant, is a good example of the consequence of aberrant hormone concentrations in seeds. In this section, the mechanisms of hormone level regulation and signal transduction are discussed.

19.3.1 ABA and GA Biosynthesis and Deactivation

ABA is a sesquiterpene hormone that maintains seed dormancy and inhibits seed germination, while GA is a diterpene hormone that releases seed dormancy and induces seed germination. Endogenous levels of active ABA and GA are determined by the relative rates of synthesis and conversion into inactive forms (Yamaguchi et al. 2007). In the past years, our understanding of genes encoding ABA and GA biosynthesis and deactivation enzymes has been greatly advanced through genetic approaches using mutants in Arabidopsis and crop species such as maize, barley, and rice. The synthesis of ABA and GA starts with geranylgeranyl-diphosphate (GGDP), a common precursor for both. ABA and GA precursors are eventually converted to the active forms as a result of a series of reactions (Crozier et al. 2000). Since the ABA and GA biosynthesis pathways have been well summarized in recent reviews (Nambara and Marion-Poll 2005; Yamaguchi et al. 2007; Yamaguchi 2008), the details will not be described here. Instead, only key events related to seed germination biology will be presented.

The key regulatory step of ABA biosynthesis, which is catalyzed by 9-*cis*-epoxycarotenoid dioxygenases (NCEDs), is the cleavage of 9-*cis*-epoxycarotenoids to produce xanthoxin (Nambara and Marion-Poll 2005; Lefebvre et al. 2006). After the discovery of *VP14*, a maize *NCED* (Tan et al. 1997), many genes encoding this enzyme have been isolated in different agricultural species including tomato (*Solanum lycopersicon*; Burbidge et al. 1999), potato (*Solanum tuberosum*; Destefano-Beltrán et al. 2006), avocado (*Persea americana*; Chernys and Zeevaart 2000), and orange (*Citrus sinensis*; Rodrigo et al. 2006). In *Arabidopsis*, *AtNCED6* and *AtNCED9* are specifically associated with ABA biosynthesis in seeds, while *AtNCED3* is responsible for ABA biosynthesis associated with stress responses (Iuchi et al. 2001). *AtNCED6* is expressed exclusively in the endosperm in developing Arabidopsis seeds. *AtNCED9* is detected in both the embryo and endosperm (Lefebvre et al. 2006). While *Atnced6* and *Atnced9* mutants do not show an ABA-deficient phenotype in vegetative tissues, seeds of these mutants exhibit

PAC-resistant germination, a characteristic typical of ABA-deficient mutant seeds. Since germination is determined by the GA-ABA hormonal balance, ABA deficiency reduces the requirement for GA, and therefore seeds become more resistant to the GA biosynthesis inhibitor compared to wild-type seeds. These results suggest that *AtNCED6* and *AtNCED9* are the major genes associated with seed maturation and dormancy in Arabidopsis, although other *NCEDs* might also be involved in these processes. Overexpression of *LeNCED1* in tomato increased ABA levels in seeds that were more dormant than wild-type seeds (Thompson et al. 2000).

Seed dormancy can be broken by fluridone or norflurazon, carotenoid synthesis-inhibiting herbicides that block downstream ABA biosysnthesis, suggesting that ABA biosynthesis is required for seed dormancy (Le Page-Degivry et al. 1990; Steinbach et al. 1997; Yoshioka et al. 1998). The hyperdormancy of *LeNCED1*-overexpressing tomato seeds mentioned above is also released by norflurazon (Thompson et al. 2000). Therefore, terminating ABA biosynthesis is a critical step for the induction of germination. Another step critical for the reduction in ABA levels is the conversion of active ABA to inactive forms (ABA deactivation). In nondormant Arabidopsis seeds, ABA levels are reduced shortly after the start of imbibition. ABA deactivation plays an important role during this period (Kushiro et al. 2004). ABA can be deactivated through hydroxylation or conjugation (Nambara and Marion-Poll 2005). A cytochrome P450 monooxygenase (ABA 8′-hydroxylase) oxidizes the C-8′ position of the methyl group of active ABA, and converts it to a less active form. Arabidopsis *CYP707A2* encodes an ABA 8′-hydroxylase that is responsible for the rapid decrease in ABA levels during seed imbibition. *cyp707a2* mutant seeds contain six-fold higher ABA levels compared to wild-type seeds, and exhibit hyperdormancy (Kushiro et al. 2004). These results suggest that in addition to the reduction in ABA biosynthesis, ABA deactivation is an essential step for dormancy release.

GA is an important antagonist to ABA in terms of seed germination control. There are many different GA molecules in plants, but only a few of these, such as GA_1 and GA_4, are active in physiological processes (Crozier et al. 2000). In Arabidopsis seeds, GA_4 is the major active GA. This active form is produced from its precursor GA_9 by the action of GA 3-oxidase, which is the final reaction and the rate-limiting step in the GA biosynthesis pathway. Major genes encoding this enzyme for seed germination-related processes in Arabidopsis are *GA3ox1* and *GA3ox2* (Yamaguchi et al. 1998). Seeds lacking either of these two genes can still germinate. However, *ga3ox1 ga3ox2* double mutant seeds do not germinate, indicating that both genes function in seed germination in a redundant manner (Mitchum et al. 2006). As is the case for ABA, deactivation also contributes to the regulation of GA levels in seeds. The major enzyme associated with GA deactivation in Arabidopsis seeds is GA 2-oxidase, which converts the active GA_4 molecule to an inactive GA_{34} form (Yamaguchi et al. 2007). The *GA2ox2* gene encoding this enzyme is upregulated under conditions where germination is suppressed (Oh et al. 2006), suggesting that GA deactivation is an important factor in controlling seed germination.

19.3.2 ABA-GA Balance, and Its Regulation by Light and Temperature

The resistance of the *Atnced* ABA biosynthesis mutant seeds to the GA biosynthesis inhibitor PAC described above indicates that less than the normal concentration of GA is sufficient to induce germination when the ABA content in seed is reduced. This strongly supports the idea of the hormone balance theory (Karssen and Lacka 1986; Karssen and Groot 1987). However, the mechanisms of the antagonistic functions between ABA and GA in seed dormancy and germination have puzzled seed biologists for many years.

Recent studies on the regulatory mechanisms of ABA and GA metabolism have started to address this long-lasting question in seed biology. One of the most significant discoveries is that ABA modulates GA levels in seeds by affecting the GA biosynthesis and deactivation pathways. In *aba2-2*, an ABA-deficient mutant, the expression of GA biosynthesis genes (*AtGA3ox2*, *AtGA20ox1*, *AtGA20ox2*, and *AtGA20ox3*) in seeds is elevated, suggesting that the expression of these GA biosynthesis genes is normally suppressed by ABA. In contrast, the expression of *GA2ox6*, a GA deactivation gene, is downregulated in *aba2-2* seeds, suggesting the promotive effect of ABA on GA deactivation (Seo et al. 2006). These results support the idea that ABA regulates GA levels in seeds (Fig. 19.1).

Another significant discovery is that ABA concentrations are regulated by phytochromes (Phy), important photoreceptors of plants. Phytochromes were originally discovered through the characterization of photoreversible dormancy of lettuce seeds (Borthwick et al. 1952). Phytochromes exist in two photoreversible

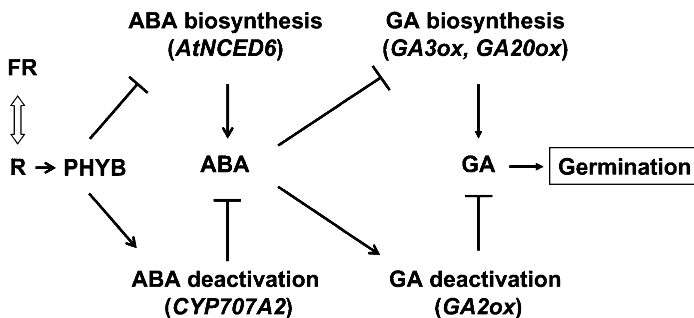

Fig. 19.1 A model showing the regulation of GA concentrations by ABA and of ABA concentrations by phytochrome. Hormonal levels are determined by a balance of biosynthesis and deactivation. ABA maintains GA concentrations low by suppressing the GA biosynthesis genes (*GA3ox* and *GA20ox*) or promoting the GA deactivation gene (*GA2ox*). ABA levels are, in turn, reduced by the suppression of the ABA biosynthesis gene (*AtNCED6*) and promotion of the ABA deactivation gene (*CYP707A2*) by phytochrome. Red light (R) promotes seed germination through the reduction of ABA concentrations, which results in an increase in GA (Seo et al. 2006). This is not the sole mechanism determining ABA-GA balance in seeds, but an example of many complex pathways. Other transcriptional control, such as downregulation of ABA biosynthesis genes by GA, is also possible. Posttranslational regulation (change at the protein level) is also important for ABA-GA balance. FR, far red light; PHYB, phytochrome B

forms, Pr (red light [R]-absorbing form), and Pfr (far red light [FR]-absorbing forms), the latter being a seed germination-promoting form. Arabidopsis seeds treated by R contain lower ABA concentrations than seeds treated by FR, which results from the downregulation of *NCED6*, an ABA biosynthesis gene, and the upregulation of *CYP707A2*, an ABA deactivation gene (Seo et al. 2006; Fig. 19.1). The changes in ABA concentrations are photoreversible, and do not occur in *phyB* mutant seeds, providing evidence for Phy involvement in ABA metabolism.

Understanding the upstream events potentially associated with hormone biosynthesis, deactivation, and signaling has also been advanced. PIF3-like 5 (PIL5 or PIF1), a basic helix-loop-helix (bHLH) protein, has been identified as a phytochrome-interacting protein in Arabidopsis seeds. This protein preferentially interacts with Pfr forms of PhyA and PhyB. *pil5* knockout seeds exhibit FR-resistant seed germination, while seeds overexpressing PIL5 are incapable of responding to R (Oh et al. 2004). Thus, PIL5 is a key component in seed light responses, and a negative regulator of seed germination. The negative effects of PIL5 on seed germination are exerted through its regulation of GA biosynthesis and deactivation. PIL5 downregulates the expression of the *GA3ox1* and *GA3ox2* GA biosynthesis genes, and upregulates the *GA2ox* GA deactivation gene, both processes reducing GA levels in seeds (Oh et al. 2006). In addition, PIL5 increases ABA concentrations in seeds by upregulating the *NCED6* and *NCED9* ABA biosynthesis genes, and downregulating *CYP707A2*, an ABA deactivation gene (Oh et al. 2007). The regulation of GA and ABA biosynthesis and deactivation genes by PIL5 is probably indirect, because PIL5 does not bind the promoter regions of those genes. PIL5 binds directly to the G-box element (CACGTG)-containing region in the promoters of *RGA* (*REPRESSOR OF ga1-3*) and *GAI* (*GIBBERELLIC ACID INSENSITIVE*), two *DELLA* repressor genes (Oh et al. 2007). *RGA*, *GAI*, and other unknown factors of which the promoters are bound by PIL5 may act upstream of GA and ABA biosynthesis. The suppression of seed germination by PIL5 in wild-type seeds is removed by R. This is achieved through the degradation of PIL5 protein by the 26S proteasome pathway, a protein degradation machinery of the cell (Oh et al. 2006).

As light stimulates seed germination, cold temperature also enhances seed germination. Incubation of imbibed seeds at low temperature (~4°C), which is called cold stratification, can release seed dormancy in many species. *GA3ox1* is induced by cold stratification (Yamauchi et al. 2004). This GA biosynthesis gene is suppressed by SPATULA (SPT), another bHLH protein. While PIL5 is degraded upon light treatment, SPT is stable under light conditions (Penfield et al. 2005). Although both SPT and PIL5 repress seed germination through the suppression of GA biosynthesis, the environmental cues controlling these two negative factors of seed germination are distinct (Tsiantis 2006).

19.3.3 ABA and GA Perception and Signal Transduction

The discovery of ABA and GA receptors in the past several years has advanced our understanding of hormone perception in plants. There are excellent review articles

for ABA (McCourt and Creelman 2008) and GA perception (Ueguchi-Tanaka et al. 2007). Here, we examine the nature of those receptors mainly in the context of seed germination. Two independent groups identified the same family of proteins in Arabidopsis as candidate ABA receptors. One group used chemical genetic approaches and identified mutants resistant to pyrabactin, a seed-selective ABA antagonist (Park et al. 2009). They isolated 12 *PYRABACTIN RESISTANCE 1* (*Pyr1*) mutant alleles. *Pyr1*, isolated by map-based cloning, encodes a member of the cyclase subfamily of the START domain superfamily. There are 13 genes in the Arabidopsis genome having marked similarity to *Pyr1*, which were named *Pyl1* to *Pyl13* (*PYR1-Like*). A separate group identified the different members of the same group of proteins through their search for proteins interacting with ABA INSENSITIVE1 (ABI1) and ABI2, type 2C protein phosphatases (PP2C) important for ABA signal transduction. These proteins were named regulatory components of ABA receptor (RCARs; Ma et al. 2009). PYR/PYL/RCARs bind ABA and inhibit PP2C activity in an ABA-dependent manner. Triple and quadruple mutants for these genes exhibit strong ABA insensitivity in seed germination (Park et al. 2009). These are probably the best candidates for seed germination-associated ABA receptors, although more research may be necessary to address the controversy about ABA receptors (Pennisi 2009).

GID1 (GIBBERELLIN INSENSITIVE DWARF1) in rice encodes a soluble receptor for GA (Ueguchi-Tanaka et al. 2005). *gid-1* mutant rice exhibits dwarfism, which is typical of GA mutants. However, this phenotype is not rescued by GA application, indicating that this is a GA-insensitive mutant (Ueguchi-Tanaka et al. 2005). Despite the lack of GA perception and subsequent signal transduction, *gid-1* rice seeds (caryopses) can still germinate. This raises the question whether GA is actually essential for seed germination. It is obvious that GA is not absolutely required for germination of seeds of cultivated rice. However, the GA requirement for germination depends on other seed germination controlling factors such as testa properties, and ABA level or sensitivity in the embryo as discussed above. In the case of rice seeds, some factors antagonizing GA function in terms of seed germination might have been unintentionally selected against during the course of domestication. Seeds of cultivated rice varieties do not have apparent pigments in the pericarp/testa, and lack dormancy. In contrast, seeds of red rice that have a pigmented pericarp exhibit deep dormancy (Cohn et al. 1983; Cohn and Hughes 1986; Gianinetti and Vernieri 2007). In Arabidopsis, *ga1-3* GA-deficient mutant seeds cannot germinate without exogenous GA; however, by introducing the *tt* mutation into the GA-deficient mutant (e.g., *ga1-3 tt4* double mutant), germination failure of the GA-deficient mutants could be reversed (Debeaujon and Koornneef 2000), suggesting that the GA requirement can be determined by the presence or absence of other factors affecting seed germination. Arabidopsis seeds lacking all three of the GA receptors, AtGIDa, AtGIDb, and AtGIDc, do not germinate, although the embryos start to grow when the seed coat is removed (Iuchi et al. 2007). Therefore, GA is an important determinant of seed germination in general.

Rice GID1 is known to interact with SLR1, a DELLA repressor protein (Ueguchi-Tanaka et al. 2005, 2007). In Arabidopsis, AtGIDs also interact with DELLA repressors, including RGL2, a well-known seed germination repressor

protein (Nakajima et al. 2006). Repression at the protein level has turned out to be a common regulatory mechanism in the GA/ABA signal transduction pathway during seed germination and postgerminative growth. RGL2 is subject to ubiquitination by SLEEPY, an F-box protein, and subsequent degradation or modification by the 26S proteasome pathway (McGinnis et al. 2003; Tyler et al. 2004). The removal of RGL2 repressor, which is a de-repression event, plays a key role in the stimulation of seed germination by GA. De-repression at the protein level is also critical for ABA signal transduction. ABI3, a key regulator of ABA signal transduction in seed maturation (Koornneef et al. 1989; Finkelstein and Somerville 1990; Nambara et al. 1991) and postgerminative seedling arrest (Perruc et al. 2007), is ubiquitinated by ABI3-INTERACTING PROTEIN2 (AIP2), an E3 ubiquitin ligase, and is subject to degradation by the 26S proteasome pathway (Zhang et al. 2005). Likewise, ABI5, which is also involved in postgerminative developmental arrest during stand establishment (Lopez-Molina et al. 2001), is removed by ABI FIVE-BINDING PROTEIN (AFP) through ubiquitin-mediated degradation (Lopez-Molina et al. 2003). Posttranslational regulation resulting in changes at the protein level plays a critical role in the regulation of seed germination and seedling growth.

19.3.4 Other Hormones

Other plant hormones are also involved in release of seed dormancy and germination. Ethylene stimulates germination by removing primary dormancy in peanut, apple, sunflower, redroot pigweed, cocklebur, and lambsquarters (KeCpczynski and KeCpczynska 1997; Matilla 2000). Seeds that fail to germinate due to unfavorable conditions after release from primary dormancy may enter into a secondary dormancy. Cocklebur, lettuce, sunflower, and *Amaranthus* seeds can be released from secondary dormancy and coaxed to germinate with ethylene or ethephon (KeCpczynski and KeCpczynska 1997).

The interactions between GA and ethylene during germination have been studied in *Fagus sylvatica*. GA_3 and ethephon can accelerate dormancy release and germination of *Fagus* seeds, while PAC and AOA (2-aminoxyacetic acid, an inhibitor of ethylene biosynthesis) delay germination (Calvo et al. 2003). Transcript levels of *FsGA20ox1* are low in the presence of GA and ethephon, but are greatly increased in the presence of PAC and AOA (Calvo et al. 2004b). These results support the presence of negative feedback regulation of *FsGA20ox1* by GA, but more interestingly they support the role of ethylene in the regulation of GA biosynthesis (Calvo et al. 2004b). Furthermore, GA_3 can reverse inhibition by AOA, and ethephon can reverse inhibition by PAC, further support of crosstalk between GA and ethylene pathways. Similarly, transcript levels of *FsACO1*, which codes for ACC (1-aminocyclopropane-1-carboxylic acid) oxidase involved in the last step of ethylene biosynthesis, are increased when seeds are treated with GA_3 or ethephon (Calvo et al. 2004a). These findings are consistent with earlier results obtained with Arabidopsis mutants: ethylene stimulates germination of *ga1*, GA-deficient mutants (Karssen et al. 1989), and GA stimulates germination in Arabidopsis ethylene receptor mutants (*etr1*; Bleecker et al. 1988). The *etr1* mutants are ethylene

insensitive, and exhibit greater dormancy than seeds of wild-type Arabidopsis (Chiwocha et al. 2005). The *etr1* mutants have greater concentrations of ABA, probably due to reduced formation of the ABA glucose ester, which probably also contributes to the increased seed dormancy. Auxin and cytokinin concentrations were also altered in the *etr1* mutants compared to the wild-type seeds (Chiwocha et al. 2005).

In sugar beet, the ratio of the ethylene precursor, ACC, to ABA increased during the early stages of germination. ACC is able to promote radicle emergence, while an ethylene inhibitor, 2,5-Norbornadiene, inhibits germination (Hermann et al. 2007), indicating that ethylene or ACC is essential for germination. When ABA is permeated into dry fruits, or when seeds are incubated on media with ABA, radicle emergence is delayed. ACC is able to partially overcome the inhibitory effect of ABA, but GA cannot, suggesting the importance of ethylene in germination of sugar beet seeds. In contrast to other species, ABA appears to induce *ACC* and *ACO* transcript accumulation in sugar beet seeds, and the ABA concentration in seeds appears to be partially regulated by a rather unique embryo-mediated ABA extrusion system (Hermann et al. 2007).

Brassinosteroid (BR) hormones were shown to promote germination in tobacco, rice, *Orobranche minor*, and *Striga asiatica* seeds (Kucera et al. 2005). BRs are also able to partially rescue the germination of severe Arabidopsis GA biosynthetic mutants (*ga1*, *ga2*, *and ga3*), and the GA-insensitive mutant *sleepy1* (Steber and McCourt 2001). Arabidopsis mutants deficient in BR biosynthesis (*det2-1*, *deetiolated 2*) or BR response (*bri1-1*, *brassinosteroid insensitive 1*) are more sensitive to ABA than are wild-type seeds during germination (Steber and McCourt 2001). BRs and GA are effective in breaking dormancy and stimulating germination of tobacco seeds under different conditions. GA is able to release photodormancy in dark imbibed seeds (Steber and McCourt 2001), while BRs do not. BRs accelerate endosperm rupture in tobacco seeds imbibed under both light and dark conditions, while GA is effective only under dark conditions.

GCR1 (*G COUPLED RECEPTOR 1*) plays an important role in both GA and BR regulated seed germination. *gcr1* mutants are less sensitive to GA and BR in seed germination (Chen et al. 2004), and when *GCR1* is overexpressed there is a loss of seed dormancy (Colucci et al. 2002; Chen et al. 2004). This suggests that GA and BRs may share a common signaling pathway, but they probably have distinct pathways as well. Studies with Arabidopsis mutants have helped to untangle some of the interactions between the various hormones that are necessary to release seeds from dormancy and promote seed germination. More detailed information concerning the hormonal crosstalk in seed germination has been summarized in the recent review by (Feurtado and Kermode 2007).

19.4 Germination Determinants Other than Hormones

Hormonal regulation is undoubtedly the major mechanism controlling seed germination. However, it is also important to find seed dormancy- and germination-associated genes independent of, or loosely controlled by, hormone pathways,

especially for application in biotechnology. Many seed germination mutants isolated to date are related to hormone biosynthesis and response. Germination phenotypes of seeds from these mutant plants are informative in terms of understanding the basic mechanisms. However, these mutants have pleiotropic effects such as wilty leaves and elongated hypocotyls. Therefore, modification of these genes cannot be applied directly to breeding programs or biotechnology practices. To address these problems, efforts have been made to identify genes that function specifically in seed dormancy and germination.

The *DELAY OF GERMINATION 1* (*DOG1*) gene involved in the control of seed dormancy has been identified by quantitative trait locus analysis of a recombinant inbred line population derived from a cross between the laboratory strain Landsberg *erecta* (L*er*), with low seed dormancy, and the dormant Cape Verde Islands (Cvi) accession (Alonso-Blanco et al. 2003). The *DOG1* gene was isolated by a combination of positional cloning and mutant analysis, and is a member of a small plant-specific gene family of unknown molecular function (Bentsink et al. 2006). *DOG1* expression is seed-specific, detectable in seeds 9 days after pollination, reaches its highest level during the last phases of seed development, and disappears upon imbibition of after-ripened seeds. These results suggest that DOG1 functions only in the establishment of dormancy during seed maturation. A *dog1* mutant does not show any obvious pleiotropic effects, except that the seeds cannot be stored at room temperature as long as wild-type seeds (Bentsink et al. 2006). Therefore, *DOG1* is a seed dormancy-specific gene that, unlike other hormone biosynthesis and response genes, provides an important agronomical trait.

Another locus important for seed germination, *reduced dormancy4* (*rdo4*), has been identified (Peeters et al. 2002). Characterization of the *rdo4* mutant identified the *MONOUBIQUITINATION1* (*HUB1*) gene that encodes a C3HC4 RING finger protein, and probably functions as an E3 ligase. This gene is required for histone H2B monoubiquitination and probably affects gene transcription. Mutation in the anther *HUB* gene (*hub2* mutant) also caused reduced seed dormancy, suggesting that chromatin remodeling may serve a general function in the induction of seed dormancy (Y. Liu et al. 2007).

There is increasing evidence that lipid mobilization plays a critical role in seed dormancy and germination. In Arabidopsis, defects in some β-oxidation enzymes cause hyperdormancy seed phenotypes (Penfield et al. 2007). Mutations in *COMATOSE* (*CTS*), an ABC (ATP-binding cassette) transporter protein that may be involved in the transport of fatty acid or acyl-CoA into peroxisomes where β-oxidation occurs, causes a forever dormant phenotype without affecting embryo morphology (Russell et al. 2000; Footitt et al. 2002). Partial mobilization of seed reserves seems to be important for dormancy release and germination induction, although these effects may be through the biosynthesis of signaling molecules, rather than fuel supply (Penfield et al. 2007).

This chapter was not intended to be comprehensive, but is focused on the basic information that is necessary to understand the frontier of seed germination research. There are other significant discoveries that have advanced seed germination biology but have not been mentioned here due to page limitations. Publications

Table 19.1 Other key publications in recent seed germination research

Category	Brief description	References
Seed proteome	Arabidopsis	Gallardo et al. (2001)
	Brassica oleracea	Soeda et al. (2005)
Seed transcriptome	Arabidopsis	Ogawa et al. (2003), Yamauchi et al. (2004)
	Medicago truncatula	Buitink et al. (2006)
Cell cycle	D-type cyclins	Masubelele et al. (2005)
GA metabolism	GA methyltransferase (GAMT)	Varbanova et al. (2007)
GA signal transduction	AP2 transcription factor	Ward et al. (2006)
	DELLA repressors	Cao et al. (2006), Willige et al. (2007), Zentella et al. (2007)
ABA metabolism	Deactivation	Okamoto et al. (2006)
ABA signal transduction	SNF-related kinase	Bradford et al. (2003), Fujii et al. (2007), Lu et al. (2007)
	Phosphatase	Gonzalez-Garcia et al. (2003), Saez et al. (2004), Miao et al. (2006), Nishimura et al. (2007), Pernas et al. (2007)
	Phosphatidic acid	Katagiri et al. (2005)
	G-protein	Ullah et al. (2002), Lapik and Kaufman (2003), Chen et al. (2006), Pandey et al. (2006), Rosnoblet et al. (2007)
	Chromatin remodeling	Dean Rider et al. (2003), Perruc et al. (2007)
	Sugar	Price et al. (2003)
	Phytochrome	Duque and Chua (2003)
	Farnesylcysteine lyase	Crowell et al. (2007)
	Nuclear transport	Verslues et al. (2006), Moes et al. (2008)
	Calcium	Zhu et al. (2007)
	DEAD-box RNA helicase	Kant et al. (2007)
Reactive oxygen species	Protein oxidation	Job et al. (2005), Oracz et al. (2007)
	Nitric oxide	Bethke et al. (2007)
Vitamins	NAD, vitamin B3 precursor	Hunt et al. (2007)
	Vitamin E	Sattler et al. (2004)
Developmental control	DAG1, 2	Gualberti et al. (2002)
Reserve mobilization	ABC transporter COMATOSE	Footitt et al. (2002), Carrera et al. (2007)
	Peroxisomal citrate synthase	Pracharoenwattana et al. (2005)
	TAG lipase SDP1	Eastmond (2006)
	ABI4, ABI5 involvement	Penfield et al. (2006)
	No utilization	Andre and Benning (2007)
Low-temperature germination	Pea mitochondria	Stupnikova et al. (2006)
	CTG gene	Salaita et al. (2005)
High-temperature germination	Lettuce	Sawada et al. (2008)
	ABA-GA at high temp.	Toh et al. (2007)
microRNA	miR159	Reyes and Chua (2007)
	miR160	P.-P. Liu et al. (2007)

containing those important insights into seed germination biology are listed in Table 19.1 for further reading.

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Chapter 20
Apomixis in the Era of Biotechnology

E. Albertini, G. Barcaccia, A. Mazzucato, T.F. Sharbel, and M. Falcinelli

20.1 Introduction

The adaptive success of living organisms depends on the maintenance of a dynamic equilibrium between creating new genetic combinations and fixing those which are more adapted to the present environment. Sexual reproduction is universally the main route to recombine genes in the short term; on the other hand, different strategies have been adopted to fix the genetic composition of individuals demonstrating high fitness. In plants, genotypes may be "immortalized" via vegetative propagation or "photocopied" by selfing of highly homozygous individuals. A third, more technically sophisticated pathway is represented by the implementation of apomixis, where a functional sexual machine is short-circuited to asexually produce embryos with the fixed genotype of the mother plant. This developmental sophistication represents a challenging research field for the reproduction biologist and a desirable trait for the plant breeder to be used in seed production schemes of elite varieties.

E. Albertini and M. Falcinelli
Department of Applied Biology, University of Perugia, Borgo XX Giugno 74, 06121 Perugia, Italy
e-mail: emidio.albertini@unipg.it

G. Barcaccia
Genetics Laboratory, Department of Agronomy and Crop Science, University of Padova, Viale dell'Università 16, 35020 Legnaro, (Padova), Italy

A. Mazzucato
Department of Agrobiology and Agrochemistry, University of Tuscia, Via S.C. de Lellis snc, 01100 Viterbo, Italy

T.F. Sharbel
Apomixis Research Group, Department of Cytogenetics, Institut für Pflanzengenetik und Kulturpflanzenforshung, Corrensstrasse 3, 06466 Gatersleben, Germany

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20.2 General Definitions and Apomixis Mechanisms

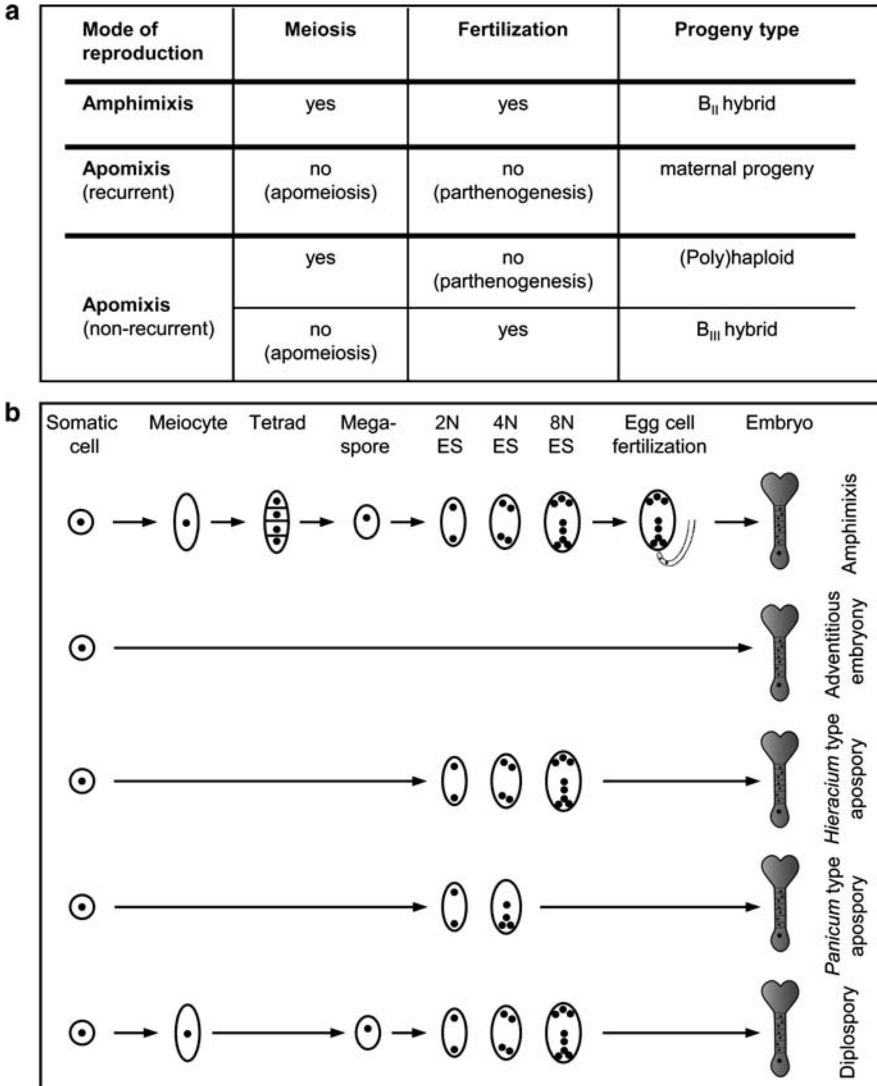
The phenomenon of apomixis, its cyto-embryological pathways and the perspective of using apomixis as a means for cloning plants by seeds have been reviewed extensively in the last decade (Savidan 2000, Savidan et al. 2001; Spillane et al. 2001; Grimanelli et al. 2001a; Koltunow and Grossniklaus 2003; Bicknell and Koltunow 2004; Ozias-Akins 2006; Hörandl and Paun 2007). However, for many aspects, the most comprehensive dissertations of apomixis terminology, mechanisms and evolution rely on a few key reviews published some 20–25 years ago (Asker 1980; Marshall and Brown 1981; Nogler 1984a; Bashaw and Hanna 1990; Asker and Jerling 1992; Koltunow 1993).

As apomictic reproduction entails the development of an embryo from a cell with a somatic chromosome number, several ways exist to produce embryos of apomictic origin. The simplest pathway avoids the production of a gametophyte, and a maternal embryo originates from one or more somatic cells of the ovule. This process is known as adventitious embryony, and can be either nucellar or integumental, depending on the tissue from which the embryogenetic somatic cell differentiates. Adventitious embryony seems to have evolved more frequently in tropical than in temperate flora. Moreover, it is more represented in diploid species, whereas other forms of apomixis are more frequent in polyploids. Among the agriculturally important species, adventitious embryony is found in several *Citrus* species, in mango (*Mangifera indica*) and in orchids. The most comprehensive treatise on adventitious embryony was published by Naumova (1992).

When the maternal embryo originates from a diploid egg cell differentiated in an unreduced embryo sac, the apomictic pathway is referred to as gametophytic apomixis. Sexual reproduction is based on the alternation of a diploid (sporophytic) and haploid (gametophytic) generation, both of which are bounded by events entailing a shift in ploidy, namely meiosis and fertilization. In gametophytic apomixis, both edge events are short-circuited; the gametophytic generation proceeds with the maternal ploidy level (and genomic composition) and the embryo is generated without the contribution of a male gamete (Fig. 20.1a). More specifically, meiosis is altered or omitted, and 2n female gametophytes and gametes are formed (apomeiosis) which then undergo embryogenesis autonomously without fertilization by a male gamete (diploid parthenogenesis).

As this combination maintains the original ploidy and may theoretically be indefinitely reiterated, it was referred to as recurrent apomixis (Nogler 1984a). In fact, the sexual program may be short-circuited in only one of the two fundamental steps; thus, it may happen that a reduced egg cell develops in the absence of fertilization, giving rise to a (poly)haploid individual (haploid parthenogenesis). In its commonest occurrence, haploid parthenogenesis takes place from the egg cell (gynogenesis); more rarely, a haploid embryo develops autonomously from a sperm nucleus (androgenesis). Conversely, the partial short-circuiting of sexual reproduction may affect only the meiotic step. Thus, an unreduced egg cell may be fertilized by a reduced male gamete, giving rise to a 2n+n hybrid or "B_{III} hybrid" (Rutishauser 1948),

rather than normal n+n (B_{II}) hybrids (Fig. 20.1a). All these pathways may occur concurrently in the same taxon and even within the same plant, as in *Poa pratensis* (Grazi et al. 1961; Barcaccia et al. 1997) and *Hieracium* (Bicknell et al. 2003), amongst others (Fig. 20.1a).



a

Mode of reproduction	Meiosis	Fertilization	Progeny type
Amphimixis	yes	yes	B_{II} hybrid
Apomixis (recurrent)	no (apomeiosis)	no (parthenogenesis)	maternal progeny
Apomixis (non-recurrent)	yes	no (parthenogenesis)	(Poly)haploid
	no (apomeiosis)	yes	B_{III} hybrid

Fig. 20.1 a Different combinations in the occurrence of meiosis and parthenogenesis give rise to sexual and asexual pathways in plant reproduction. **b** Schematic representation of female sporogenesis and gametogenesis in sexual plants and short-circuited alternative pathways in the most common forms of apomixis

Because haploid parthenogenesis and genome accumulation, an alternative term given to the occurrence of B_{III} hybrids (Leblanc and Mazzucato 2001), entail shifting of the original ploidy level, they cannot reiterate themselves and, for this reason, have been referred to as "non-recurrent apomixis" (Nogler 1984a). Although not offering a stable means for genotype propagation, non-recurrent apomixis has likely been an important player in the evolution of polyploid species and is regarded as a useful tool to scale-up or -down the chromosome number in breeding programs.

20.3 Embryological Pathways of Gametophytic Apomixis

In gametophytic apomixis, the unreduced embryo sac may arise from a somatic nucellar cell which acquires the developmental program of a functional megaspore, a mechanism referred to as apospory. Alternatively, if the embryo sac forms from a megaspore mother cell with suppressed or modified meiosis, the pathway is called diplospory (Fig. 20.2b). These two pathways, leading to the production of 2n egg cells and broadly referred to as apomeiotic pathways, offer a variety of different developmental schemes which have been the object of several descriptions and reviews (Nogler 1984a; Asker and Jerling 1992; Crane 2001).

In apospory, when the unreduced embryo sac develops into an eight-nucleate, seven-celled gametophyte (similar to the *Polygonum*-type found in sexuals), this is referred to as *Hieracium*-type apospory (Fig. 20.1b). This scheme was first described in *Hieracium* a century ago (Rosenberg 1907) but, subsequently, it was found in other Compositae (e.g. *Crepis*) and in the Poaceae (e.g. *P. pratensis* and *Hierochloe* spp.), also in genera belonging to different families, such as *Hypericum*, *Ranunculus* and *Beta* (reviewed in Nogler 1984b). Usually one or, more rarely, a few aposporic initials differentiate from one or more nucellar cells which are in contact with the differentiated meiocyte or its derivatives, and which enlarges to form a large vacuole at the chalazal pole. This represents the best moment to recognize aposporic activity in species with *Hieracium*-type apospory (Fig. 20.2a), as mature embryo sacs of sexual or apomeiotic origin are difficult, if not impossible, to differentiate. However, a frequent occurrence inside the same ovule is the development of both the reduced and the unreduced embryo sac to produce multiple mature gametophytes and polyembryony in the seed.

In an alternative pathway, the aposporic initial cell behaves as in the former case but gametogenesis involves only two free divisions, resulting in a mature four-nucleate, four-celled embryo sac. This so-called *Panicum*-type embryo sac shows a three-celled egg apparatus, a single unreduced polar nucleus and no antipodals (Fig. 20.1b). *Panicum*-type apospory is frequent in the Paniceae (genera *Brachiaria*, *Cenchrus*, *Eriochloa*, *Panicum*, *Paspalum*, *Pennisetum* and *Urochloa*) and Andropogoneae (the *Bothriochloa*-*Dichanthium*-*Capillipedium* agamic complex and the genera *Chloris*, *Heteropogon*, *Hyparrhenia*, *Sorghum* and *Themeda*)

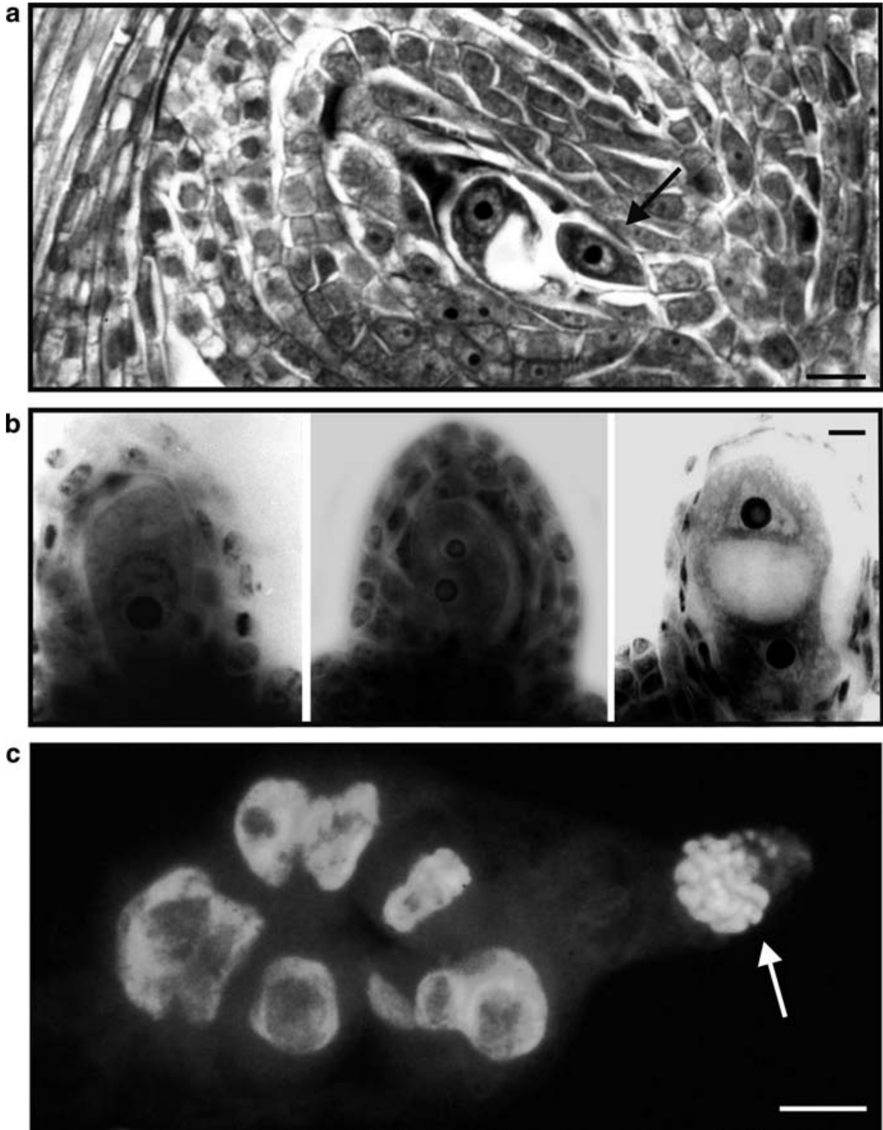


Fig. 20.2 **a** Occurrence of an aposporic initial (*arrow*) in Poa pratensis. **b** Diplospory in the TNE Medicago falcata mutant: unreduced FDR-type monad due to omitted or modified meiosis I (*left*), binucleated (*centre*) and non-polarized ES, and polarized ES containing unreduced nuclei (*right*), and **c** embryo developed to the globular stage (*arrow*) before fertilization of polar nuclei in *P. pratensis*. Bar¼10 mm

tribes of the Poaceae (Nogler 1984a; Bashaw and Hanna 1990). As in the *Hieracium* type, in these species the legitimate lineage may also develop alongside the aposporic initials. However, it appears that normally all sexual megaspores

degenerate. In this intra-ovular competition, it seems that the timing of differentiation of aposporic initials is the crucial factor; the earlier the differentiation, the higher the competitive superiority of apospory versus sexuality. For scoring apospory, the *Panicum*-type pathway allows recognition of the unreduced embryo sac also at maturity. Although the model for apospory development is strictly species-specific, cases have been reported of *Paspalum* species in which both four- and eight-nucleate embryo sacs are found (Reusch 1961; Quarin et al. 1982).

Diplospory offers a richer repertoire of developmental pathways. When meiosis is completely bypassed, it is referred to as *Antennaria*-type or mitotic diplospory. Mitotic diplospory is widely distributed, e.g. in *Antennaria*, *Eupatorium*, *Poa alpina*, *Parthenium*, *Eragrostis* and *Tripsacum* (reviewed in Nogler 1984a) and, owing to the absence of meiosis, it represents a form of apomeiosis which fully guarantees the fixation of the maternal genome (Figs. 20.1b, 20.2b).

In the *Taraxacum*-type diplospory (aneuspory), by contrast, meiosis begins but is largely asynaptic (cf. absence of pairing between homologous chromosomes) and a restitution nucleus in the first division produces a dyad of unreduced megaspores. The chalazal megaspore generally produces the unreduced embryo sac. Whether the maternal genome is fixed or not depends on whether any bivalents have formed and crossover has taken place. In addition to *Taraxacum*, this type of diplospory has been found in *Erigeron*, *Boechera* (formerly *Arabis*), *Agropyrum* and some *Paspalum* species (reviewed in Nogler 1984a). In other diplosporic pathways (*Ixeris*, *Datura* and *Allium* types), the first meiotic division is carried out and the unreduced megaspores present the maternal genome with the effects of recombination. These pathways have been described in detail by Asker and Jerling (1992). In contrast to what happens in adventitious embryony and apospory, in diplospory the occurrence of more than one embryo inside the same seed (polyembryony) is, theoretically, excluded.

Irrespective of how the unreduced embryo sac has formed, the second component of (recurrent) gametophytic apomixis consists of autonomous egg cell development in the absence of fertilization (diploid parthenogenesis). In sexual species, diploid parthenogenesis occurs rarely and is called matromorphy, examples of which have been reported mainly in *Brassica* but also in *Fragaria*, *Raphanobrassica* and other species (reviewed in Asker and Jerling 1992). It is thought that the mechanisms controlling diploid parthenogenesis are not different from those responsible for the autonomous development of a reduced egg cell. However, compared to apomeiosis, the study of parthenogenetic mechanisms has received less attention.

The third (and last) component for the production of a functional apomictic seed is functional endosperm formation. In many apomicts, the endosperm is initiated autonomously without any contribution from male gametes. Autonomous endosperm development is widely spread in the Compositae and common among species showing diplospory and adventitious embryony. Endosperm ploidy may vary depending on whether or not the unreduced polar nuclei fuse before initiating

the endosperm divisions. In autonomous species, the synergids remain intact and the male organs are often not functional.

More frequently, endosperm development requires a pollination stimulus to occur (pseudogamy). This is found more commonly in aposporic species, including the families Rosaceae and Poaceae. It is rare in the Compositae. The pathway for endosperm formation is usually conserved at the genus level. Pseudogamy may be characterized by simple pollen tube growth in the style, cytoplasmic penetration or, most frequently, true fertilization of the polar nuclei in the embryo sac. In some pseudogamous species, parthenogenetic development precedes secondary fertilization to give rise to precocious embryony (or proembryony; Fig. 20.2c). In pseudogamous species, the endosperm ploidy level is more variable than in autonomous species; the expected level of the 4(n) maternal:1(n) paternal ratio is often altered by the variable number of polar nuclei involved, the extent of their fusion, and the number and ploidy level of the male gamete(s) which operate fertilization. Thus, in many cases the 2:1 maternal-to-paternal ratio of the endosperm is maintained, as is the case for *Panicum*-type apospory in which a single 2n polar nucleus is fertilized by a reduced male gamete) or, in *Hieracium*-type apospory, if the polar nuclei do not fuse and are each fertilized by a single sperm cell.

20.4 Genetic and Epigenetic Control of Apomixis

Several models for the genetic basis of apomixis have been proposed, including divergence in the number of genes, their function and allelic relationships, and dominance over sexuality (Asker and Jerling 1992; Koltunow et al. 1995; Carman 1997; Grimanelli et al. 2001a; Noyes 2005). Genetic analysis in several species (Table 20.1) has consistently demonstrated a simple inheritance system and a few Mendelian genes controlling the expression of apomixis or its components (Barcaccia et al. 2000; Grimanelli et al. 2001a; Bicknell and Koltunow 2004; Catanach et al. 2006; Schranz et al. 2006; Noyes et al. 2007). By contrast, molecular and cytogenetic analyses of the chromosomal region(s) carrying the determinants of apomixis in several species have unveiled attributes indicative of a complex genetic control and/or a system of polygenes, in addition to mechanisms involving lack of recombination, transacting elements for gamete elimination, supernumerary chromatin structures and DNA rearrangements (Leblanc et al. 1995a; Grimanelli et al. 1998; Roche et al. 1999; Noyes and Rieseberg 2000; Goel et al. 2003; Matzk et al. 2005; Calderini et al. 2006).

Recent data collected in species forming seeds through distinct asexual pathways, such as *Hieracium* spp., *P. pratensis* and *Tripsacum dactyloides*, suggest that gametophytic apomixis relies upon either spatial or temporal misexpression of genes acting during female sexual reproduction (Grimanelli et al. 2003; Tucker et al. 2003; Albertini et al. 2004). However, although genes showing differences in spatial and

Table 20.1 Genetic inheritance and molecular mapping of apomixis components (apomeiosis and parthenogenesis). Genetic models are based on the segregation analysis of progenies from crosses between sexual and apomictic genotypes and are supported by the co-segregation of tightly linked molecular markers

Species	Type of apomixis	Endosperm development	Parental ploidy and type of crosses[a]		Deduced genotypes	Linked markers	Suppressed recombination	Main references
Apomeiosis								
Ranunculus auricomus	Apospory	Pseudogamous	2x–4x	Intrageneric	Aaaa	–	–	Nogler (1984a)
Panicum maximum	Apospory	Pseudogamous	$4x_i$–4x	Intraspecific	Aaaa	–	–	Savidan (1982)
Pennisetum squamulatum	Apospory	Pseudogamous	$4x_i$–6x	Intrageneric	Aaaaaa	0 cM	Yes	Ozyas-Akins et al. (1998)
Brachiaria brizantha	Apospory	Pseudogamous	$4x_i$–4x	Intrageneric	Aaaa	1.2 cM	No	Pessino et al. (1998)
Paspalum simplex	Apospory	Pseudogamous	$4x_i$–4x	Intraspecific	Aaaa	0 cM	Yes	Labombarda et al. (2002)
Hieracium spp.	Apospory	Autonomous	2x–3x	Intrageneric	Aaa	–	–	Bicknell and Koltunow (2004)
Hypericum perforatum	Apospory	Pseudogamous	2x–4x	Intraspecific	Aaaa	0 cM	–	Barcaccia et al. (2007)
Poa pratensis	Apospory	Pseudogamous	4x–5x	Intraspecific	Aaaaa	–	–	Albertini et al. (2007)
Tripsacum dactyloides	Diplospory	Pseudogamous	2x–4x	Intergeneric	Dddd	0 cM	Yes	Grimanelli et al. (1998)
Erigeron annuus	Diplospory	Autonomous	2x–3x	Intrageneric	Ddd	0 cM	Yes	Noyes and Rieseberg (2000)
Taraxacum officinale	Diplospory	Autonomous	2x–3x	Intraspecific	Ddd	0.2 cM	No	Van Dijk and Bakx-Schotman (2004)
Parthenogenesis								
Poa pratensis	Apospory	Pseudogamous	4x–6/8x	Intraspecific	Pppppp	6.6 cM	No	Barcaccia et al. (1998)
Hieracium spp.	Apospory	Autonomous	2x–3x	Intrageneric	Ppp	–	–	Bicknell and Koltunow (2004)
Erigeron annuus	Diplospory	Autonomous	2x–3x	Intrageneric	Ppp	7.3 cM	No	Noye and Rieseberg (2000)
Taraxacum officinale	Diplospory	Autonomous	2x–3x	Intraspecific	Ppp	–	–	Van Dijk and Bakx-Schotman (2004)

[a] Parental ploidy: i, induced. -, not determined

temporal expression patterns between apomicts and their sexual counterparts have been reported (Pessino et al. 2001; Rodrigues et al. 2003; Albertini et al. 2005; Chen et al. 2005), their functions remain largely speculative. In the current view, gametophytic apomixis is thought to rely on three genetically independent Mendelian loci, each exerting control over a key developmental component, these being the formation of apomeiotic megaspores, the parthenogenetic capabilities of unreduced egg cells and modified endosperm development (Noyes and Rieseberg 2000; Albertini et al. 2001a; Grossniklaus et al. 2001a; Koltunow and Grossniklaus 2003; Bicknell and Koltunow 2004; Vijverberg and van Dijk 2007).

The importance of parent-of-origin effects and, more generally, of epigenetic factors during sexual reproduction and early seed development has emerged recently in apomicts (Guitton and Berger 2005; Köhler and Grossniklaus 2005; Autran et al. 2005; Takeda and Paszowski 2006; Xiao et al. 2006; Feil and Berger 2007; Nowack et al. 2007). Although Grossniklaus and Schneitz (1998) and Grossniklaus et al. (2001a) proposed that the regulation of apomixis might depend on heritable epialleles and that relaxation of genomic imprinting is a requirement at least for endosperm development in apomicts, the relevance of epigenetics in apomictic developmental patterns remains largely unexplored (Koltunow and Grossniklaus 2003; Ranganath 2004).

In most apomicts, apospory and diplospory have been proven to be simply inherited on the basis of the segregation of the trait in crosses between sexual seed parents and apomictic pollen parents. Since apomixis is always associated with hybridity and heterozygosity, segregation for the mode of reproduction as well as co-segregation of molecular markers have been studied in most species by adopting pseudo-testcross mapping strategies (Barcaccia et al. 1998; Ozias-Akins et al. 1998; Pessino et al. 1998; Noyes and Rieseberg 2000; Van Dijk and Bakx-Schotman 2004). In some species, both female (sexual) and male (apomict) genetic maps have been constructed (Porceddu et al. 2002; Jessup et al. 2003; Pupilli et al. 2004).

Apospory has rarely been shown to segregate from parthenogenesis, behaves as a dominant trait, and is inherited in a Mendelian fashion, although sometimes subject to segregation distortion (reviewed in Ozias-Akins 2006). This pattern of inheritance has been observed for *Pennisetum squamulatum* (Dujardin and Hanna 1983; Ozias-Akins et al. 1998), *Cenchrus ciliaris* syn. *Pennisetum ciliare* (Sherwood et al. 1994; Jessup et al. 2002), *Panicum maximum* (Savidan 2000; Ebina et al. 2005), *Brachiaria* spp. (do Valle et al. 1994; Miles and Escandon 1997), *Paspalum notatum* (Martínez et al. 2001), *Ranunculus* spp. (Nogler 1984b), *P. pratensis* (Albertini et al. 2001a) and *Hieracium* spp. (Bicknell et al. 2000). A more complex genetic model, advanced for the evolution of apomixis from sexual plants (Holsinger 2000), was recently postulated for *P. pratensis* which includes single, unlinked genes for initiation of apospory, apospory prevention, parthenogenesis initiation and parthenogenesis prevention, as well as a megaspore development gene (Matzk et al. 2005).

A single regulatory gene has been proposed as sufficient for the induction of apomixis (Peacock 1992) and, although simple genetic inheritance appears to support this hypothesis, molecular evidence suggests that more complex genetic

control of the entire apomixis process cannot be discounted. In particular, the linkage groups typically transmitted with apospory display large blocks of non-recombining molecular markers, leading to speculation that adapted gene complexes within supernumerary chromatin might be required for the function of at least certain types of apomixis (Roche et al. 2001; Ozias-Akins et al. 2003). The association of apomixis with a chromosomal region lacking genetic recombination was first described in *P. squamulatum* (Ozias-Akins et al. 1998). Extensive characterization of this chromosomal region using RFLP (restriction fragment length polymorphism) markers and FISH (fluorescence in situ hybridization) of apomixis-linked clones has shown the region to be extremely large in size, heterochromatic, and highly hemizygous (Ozias-Akins et al. 1998; Roche et al. 1999; Goel et al. 2003; Akiyama et al. 2004). A heterochromatic and hemizygous region was also found in the polyploid apomicts *P. squamulatum* and *C. ciliaris*, and is indicative of heteromorphism between the homologous chromosomal pairing partners which has apparently resulted from an insertion in both species, combined with an inversion/translocation in the former (Akiyama et al. 2005). Lack of genetic recombination and hemizygosity are not confined to the genus *Pennisetum* and close relatives but have also been found in *Paspalum simplex* (Labombarda et al. 2002). The association of apospory with a heterochromatic region of the genome, rich in retrotransposons, raises the intriguing possibility that DNA structure and/or RNA interference (Lippman et al. 2004) could play a role in the control of expression of apomictic-related genes.

In *T. dactyloides* (Grimanelli et al. 2003) and *Hypericum perforatum* (Barcaccia et al. 2006), it has been shown that apomeiosis, i.e. displospory in the former and apospory in the latter, and parthenogenesis are developmentally uncoupled, supporting the hypothesis of two distinct genetic factors controlling these traits in both apomictic species. A clearly documented case of recombination between apospory and parthenogenesis is found in *P. pratensis* (Barcaccia et al. 2000; Albertini et al. 2001a, b). Independence between diplospory and parthenogenesis was also reported for *Taraxacum officinale* (Tas and van Dijk 1999; van Dijk et al. 1999; van Dijk and Bakx-Schotman 2004), where autonomous endosperm development was shown to segregate independently from diplospory and parthenogenesis (van Dijk et al. 2003; Vijverberg et al. 2004). Similarly, progeny from a cross of sexual diploid and apomictic triploid genotypes of *Erigeron annuus* showed a range of chromosome numbers which were not predictive of reproductive mode, even though a single locus model for diplospory was supported by segregation data (Noyes and Rieseberg 2000). While all parthenogenetic plants were diplosporic, several diplosporic plants were not able to form embryos, suggesting that a genetic component for parthenogenetic development of egg cells had been eliminated. Genetic mapping of the segregating population provided several AFLP (amplified fragment length polymorphism) markers linked to either diplosporic apomeiosis or parthenogenesis, providing support that the two apomixis components were segregating independently (Noyes and Rieseberg 2000).

20.5 Evolution of Apomixis and Population Genetics in Apomicts

The origin and persistence of asexual reproduction remains one of the most challenging phenomena in evolutionary biology (Bell 1982). In plants and animals, asexuality is derived from sex (amphimixis), and not only has it originated independently between different species but it has also evolved recurrently within certain species. Hence, in certain contexts, natural selection has repeatedly favoured the switch to asexual reproduction. Despite hypothesized disadvantages associated with asexual reproduction, including limited genetic diversity and mutation accumulation, asexual plants and animals are surprisingly stable from an evolutionary perspective, and thus questions regarding the origin and evolution of asexuality confront evolutionary and population biologists.

Many wild apomictic species are characterized by hybridity and polyploidy (Richards 2003) and, interestingly, these characteristics are also shared by a majority of asexual animal taxa. It is still unclear what the relative contributions of hybridization and polyploidy are to asexual lineage origin and evolution, as both phenomena can have diverse regulatory consequences (Comai et al. 2003; Osborn et al. 2003; Swanson-Wagner 2006) which could conceivably lead to coordinated deregulation of the sexual pathway in a sexual ancestor. In addition, both naturally occurring and induced mutants demonstrating the individual components separately have been identified (Curtis and Grossniklaus 2007; Ravi et al. 2008), implying that many taxa have the potential to express apomixis-like traits, in addition to supporting the hypothesis that each component is under independent regulation. The actual molecular mechanisms underlying apomixis expression are the focus of intense research described in more detail in other sections of this chapter.

Apomictic taxa are often members of "complexes" and, as the name implies, this involves species which are characterized by a complicated mixture of inter- and intraspecific genetic and phenotypic variation and gene flow. Most apomictic taxa are facultative, meaning that a single individual can produce seeds through both sexual and apomictic pathways. Furthermore, apomicts and their sexual relatives are often sympatric (but not necessarily syntopic) and morphologically difficult to differentiate. Closely related sexual and asexual taxa frequently have different, although overlapping, ranges of adaptation, referred to as "geographical parthenogenesis" (Vandel 1928). More specifically, compared to their sexual relatives, apomictic plants typically have (1) larger geographic ranges, (2) ranges which extend into more elevated latitudes and altitudes, and (3) better abilities to colonize previously glaciated regions (Bierzychudek 1987). With reference to apomictic plants, geographical parthenogenesis applies only to gametophytic apomixis and not to adventitious embryony, as the latter tends to be prevalent in tropical species and is characterized by different mechanisms and ecological constraints (Richards 1997). Apomicts differ from their sexual relatives not only in reproductive mode but, in most cases, also in ploidy (cf. apomicts are polyploid). Hence, the phenomenon of geographical parthenogenesis could equally be explained by ploidy

differences as it could by reproduction (Bierzychudek 1987). While a number of factors likely contribute to these geographic differences between amphimictic and apomictic plants, including the Pleistocene origins of apomixis in conjunction with hybridization and polyploidy, unidirectional gene flow, niche targeting by asexual clones, and limited biotic interactions in regions of glaciations, the relative influence of each is probably species-specific (Hörandl 2006).

Asexual taxa are typically thought to be better colonizers than their sexual counterparts. In animals, this has been associated with the "two-fold cost of sex", as sexuals require two individuals to reproduce (male and female), while asexuals require only one. Hence, an asexual population has the potential to grow faster (Maynard Smith 1978). Apomicts, by nature of being hermaphroditic, do not suffer this two-fold cost and, although there appears to be decreased selection pressure on the male line (Voigt et al. 2007), some functionality must nonetheless be maintained in order to fertilize the primary endosperm nuclei, i.e. pseudogamy. Within this context, apomicts can more easily found new populations (as do selfing sexual plants), since only a single individual is required, and this is reflected by the invasiveness of some apomictic taxa, e.g. *T. officinale* (Brock et al. 2005) and *H. perforatum* (Vilà et al. 2003).

A biologist who is planning a population level study of an apomictic taxon is thus faced with two problems. One must first be able to differentiate between sexual and apomictic individuals which may or may not be morphologically distinct and share similar geographic ranges. Secondly, as wild apomicts are typically facultative, assessing variations in sexual and apomictic seed production within individuals is essential for understanding population dynamics and gene flow. Assuming a difference in ploidy between apomicts and sexuals, time-consuming karyological or cell size-based analyses can be made of each collected individual. Developments in flow cytometry have facilitated analyses of ploidy on the population level and, today, literally 1,000s of individuals can be analyzed in a few days (Sharbel and Mitchell-Olds 2001). Isolating flowers which have been emasculated can be used to identify seed formation through autonomous apomixis (Richards 1997), whereas differentiating between pseudogamous apomicts and selfing individuals in this way is more difficult in cases of self-compatibility. Apomictic reproduction should be characterized by fixed heterozygosity, while selfing, which is also reproduction in the absence of cross pollination, leads to homozygous offspring. Thus, comparison of genetic markers between parents and offspring can be used in order to identify apomictic offspring having identical fixed heterozygous genotypes. Such an approach is, of course, time-consuming and potentially problematic, if seeds cannot be collected and germinated in the glasshouse. Alternatively, if one uses a "normal" diploid sexual population and its expected population genetic parameters, e.g. linkage equilibrium, random mating or Hardy-Weinberg equilibrium as a point of reference, then deviations from expected variation can be a signal that apomixis has played a part in influencing population structure (Halkett et al. 2005). For example, the identification of fixed genotypes within a population can be used to infer apomixis, although the confidence of such analyses will be influenced by the choice of genetic marker, e.g. dominant AFLPs versus codominant microsatellites (Leblanc and Mazzucato 2001; Arnaud-Haond et al. 2007).

The problem with using indirect methods to identify apomictic individuals is that they are subject to ascertainment bias. Conclusions can be erroneously drawn as a result of the type of method used to screen for apomixis, as well as the schemes employed to choose both population and tissue samples for analysis. More recently, the "flow cytometric seed screen" (FCSS; Matzk et al. 2000) has been developed as an effective, cost-efficient and rapid way to directly measure seed formation in both sexual and apomictic plants. The FCSS method uses a flow cytometric profile of individual seeds to infer the mechanisms of seed production (Matzk et al. 2000). In a normal diploid sexual plant, a reduced (C) egg cell and a reduced central cell with two polar nuclei (C+C) are fertilized by two sperm cells to form a 2C embryo and 3C endosperm. Depending upon the taxon, apomixis is characterized by the formation of reduced and/or unreduced embryo sacs which will be fertilized by reduced or unreduced sperm cells (Matzk et al. 2000). Hence, the FCSS serves to identify *deviations* from the typical sexual 2:3 embryo:endosperm ratio. Using FCSS, the dynamics of seed formation in an apomictic plant can be precisely determined, and a wide range of different reproductive pathways identified (Matzk et al. 2001; Naumova et al. 2001). Importantly, analyses of large numbers of individual seeds per plant demonstrate genotype-specific quantitative variation for sexual and apomictic seed formation (O.M. Aliyu and T.F. Sharbel, unpublished data) and, furthermore, have enabled the identification of relatively rare phenomena, e.g. autonomous endosperm formation and fertilization of the apomeiotically derived egg cell (Voigt et al. 2007). Finally, FCSS analyses of seeds harvested at different stages, i.e. immature versus mature, have also shown that an apomictic plant has the potential to produce much larger levels of variation than expected if only mature dried seeds are measured (Voigt et al. 2007). Thus, it may be useful to differentiate between primary and secondary apomixis phenomena, since most of the potential variability produced by an apomict can be truncated by downstream developmental effects which are not directly associated with apomeiosis, the first and most important step (Voigt et al. 2007). This has significant implications for ongoing genomic and transcriptomic projects whenever statistical correlations with phenotypic data are made, as imprecise assessments of quantitative variation could potentially lead to spurious associations with marker data. The use of molecular markers can thus shed light upon the presence of apomixis within populations but, more importantly and in conjunction with precise methods of measuring quantitative variation in apomixis seed production (Matzk et al. 2000), inferences can be made regarding aspects of the origin and evolution of different apomictic lineages.

Sexual reproduction is hypothesized to be advantageous, since it is a mechanism through which genetic diversity can be generated and maintained at the population level. Conversely, asexually reproducing organisms are expected to be genetically homogenous and, thus, relatively static, since evolution requires genetic variation in order to proceed. Furthermore, asexual lineages are expected to accumulate deleterious mutations each generation, and are doomed to eventual extinction, i.e. Muller's Ratchet (Muller 1964; Kondrashov 1994). A few rare cases of ancient asexuality—asexual scandals (Rice and Friberg 2007)—nonetheless exist and, while these do present challenges to accepted concepts, interesting molecular

mechanisms have been uncovered which likely counteract the effects of an absence of sex (Welch et al. 2004; Pouchkina-Stantcheva et al. 2007).

Strictly speaking, it is almost inappropriate to discuss apomixis in terms of "populations", as no gene flow sensu stricto should occur between different apomictic individuals. In many cases, the more accurate scenario is a sexual swarm from which asexual clonal lineages arise recurrently through time. From a molecular genetic perspective, an analysis of naturally occurring apomictic complexes will uncover two kinds of genetic variability. First, consider two different apomictic lineages which arise independently at different times and/or in different geographical locations. As the lineages arise from local sexual gene pools which may also vary in space and time, they will each be established with a sampling of alleles from their respective locations (or time) and, hence, exhibit differing founding genotypes. Since their first appearance, the asexual lineages will have accumulated mutations, thereby introducing a second level of variation which differentiates them. Contrasts between these two types of variation are essential for answering questions of clonal origin and longevity, and a number of analytical approaches have been taken to classify both levels using population genetic data (Mes 1998; Meirmans and Van Tienderen 2004; Halkett et al. 2005). A further confounding effect is the instability of the asexual genome, which is no longer constrained by meiosis. Hemizygosity and chromosomal heteromorphy, both of which have been described in numerous asexual plants and animals, are examples of how homologous regions on sister chromosomes can diverge physically from one another (Birky 1996). Duplications may also accumulate in asexual genomes, leading to confusing patterns of microsatellite variation, and difficulties in assuming homology of allelic size variants (Corral et al. 2008). The degree to which all such effects influence the evolution of asexuality must be considered within the context of the taxon being studied, and Halkett et al. (2005) have attempted to outline a logical approach as to how studies of asexual systems should be conducted.

Finally, as in other fields, the application of new massively parallel sequencing, transcriptomic and proteomics technologies to wild asexual taxa will undoubtedly demonstrate that asexuality is not static on the individual and population levels. Such approaches will not only help to elucidate why asexuality has remained a successful form of reproduction but, in addition, analysis of genomes which are relatively unconstrained by the effects of meiosis will contribute to our understanding of the molecular dynamics and evolution of "normal" sexual genomes.

20.6 Transferring Apomixis in Crops from Wild Relatives, Molecular Mapping of Apomixis Components and Map-Based Cloning of Candidate Genes

The transfer of apomixis into crops from wild relatives has been performed mainly in pearl millet from the aposporous *P. squamulatum* and has been attempted in maize from the diplosporous *T. dactyloides*. In a research program to transfer

apomixis from *P. squamulatum* to pearl millet, a polyhaploid plant ($2n=3x=21$) was discovered in the uniform open-pollinated progeny of an apomictic interspecific hybrid between pearl millet and *P. squamulatum*. The polyhaploid plant was shorter, less vigorous and smaller than its maternal parent. It probably originated by parthenogenetic development of a reduced egg cell in the apomictic interspecific hybrid. The polyhaploid plant was male-sterile and partially female-fertile, having multiple aposporic embryo sacs in 95% of the ovules. Seed set was as low as 3% when open-pollinated and 33% when pollinated with pearl millet, due to competition among multiple embryos developing in the same ovule. Seventeen progeny plants from seed produced under open-pollination on the polyhaploid each had 21 chromosomes and were morphologically uniform and genetically identical to the maternal parent (Dujardin and Hanna 1983, 1984).

These results demonstrated the possibility of conferring the apomictic trait to a plant which normally reproduces sexually, such as pearl millet, by introducing the desired gene(s) controlling apomixis. This information was patented as a protocol to generate an apomictic hybrid plant which produces progeny identical to itself, by transferring an apomictic mechanism from a wild species to a cultivated plant (US Patent 5811636; Ozias-Akins 1998). In particular, this procedure has been reported as exploitable for breeding apomictic pearl millet–*P. squamulatum* hybrids which are more genotypically millet-like. Seeds can be multiplied by crossing an apomictic plant with a nurse cultivar as a pollen source for endosperm formation in seeds.

The genus *Tripsacum* includes wild relatives of maize (*Zea mays* L.) widely distributed across the American continent, and highly variable in many aspects (Randolph 1970; Berthaud et al. 1997). Efforts towards allele mining from this diverse genetic reservoir have been limited, with one notable exception concerning apomixis (reviewed in Savidan 2000). Within the tribe *Maydae*, apomixis occurs only in *Tripsacum* (Brown and Emery 1958), making the genus an important candidate to elaborate strategies for transfer of apomixis to maize, either through breeding or by genetic engineering. *Tripsacum* species typically form an agamic complex (sensu Babcock and Stebbins 1938), whereby diploid individuals ($2n=2x=36$) are sexual and polyploid individuals ($2n=3x$ to $6x$) reproduce apomictically. Apomixis is diplosporic of the *Antenaria* type (Farquharson 1955; Leblanc et al. 1995b; Grimanelli et al. 2003). The megaspore mother cell "skips" meiosis and differentiates directly into a uninuclear embryo sac functional (Grimanelli et al. 2003). Further differentiation into embryo sacs resembles that of the *Polygonum* type. Activation of unreduced egg cells through unknown developmental alterations in the embryo sacs may induce embryogenesis in the absence of fertilization (Farquharson 1955; Bantin et al. 2001). However, the developmental pattern of maternal embryos is interrupted after a few rounds of mitotic divisions, resulting in quiescent proembryos within unfertilized embryo sacs (Grimanelli et al. 2003). Pollination, followed by the delivery of two sperm cells into the mature embryo sac and fertilization of the central cell only, is required for seed development. Besides the apomictic pathway, reproductive traits which allow genetic variation have been preserved through evolution in *Tripsacum*, as in many other apomicts. The most documented cases result from partial or complete restoration of

sexual programs (Asker and Jerling 1992; Bicknell and Koltunow 2004) but other mechanisms, such as incomplete nucleus restitution during meiosis abortion, mitotic and meiotic non-disjunction, somatic recombination and gene mutation, have been reported as well (Hair 1956; Richards 1996; Noyes 2005).

Diplospory was determined to be under a simple genetic control in a cross between *Z. mays* and *T. dactyloides*, and several RFLP markers, known to be positioned on the long arm of maize chromosome 6, were found to be strictly co-segregating with diplospory (Leblanc et al. 1995a; Grimanelli et al. 1998). Since the days when maize and *Tripsacum* were hybridized for the first time (Mangelsdorf and Reeves 1931), pathways for introgressing *Tripsacum* genetic material into the crop have been investigated extensively (e.g. Harlan et al. 1970; Harlan and DeWet 1977). Nevertheless, in spite of several decades of effort (Petrov et al. 1984; Leblanc 1995a, 1996; Kindiger and Sokolov 1997), maize germplasm expressing some level of apomixis has not yet been recovered. Conventional backcrossing strategies using *T. dactyloides* as an apomictic donor yielded facultative apomictic hybrids possessing two maize genomes and one genome from *T. dactyloides*, i.e. $2n=38=20+18$ (Leblanc et al. 1996). The localization of apomixis to a maize–*Tripsacum* chromosome translocation supported the conclusion that only a single *Tripsacum* chromosome transmitted apomixis (Kindiger et al. 1996).

A detailed understanding of the inheritance of apomixis in model apomicts is required for the identification of candidate genes and eventual transfer of this valuable trait into species which naturally propagate sexually. Detailed genetic mapping analysis is extremely difficult, due to the association of facultative apomixis with polyploidy and variable but elevated levels of heterozygosity. Nonetheless, the chromosomal regions associated with apomixis factors have been characterized in several species, and molecular markers tightly linked to putative apomeiosis and/or parthenogenesis loci have been identified. Molecular differential screening of plants with contrasting modes of reproduction is still considered one of the most powerful tools for identifying, mapping and isolating the gene(s) underlying the expression of apomixis. Even in remarkably complex genomes like those of apomictic species, the visualization of molecular markers in combination with bulked segregant analysis (Michelmore et al. 1991) was shown to be effective for detecting gene polymorphisms and genome sequences useful for positional cloning (Barcaccia et al. 1998; Ozias-Akins et al. 1998; Pessino et al. 1998). In the case of facultative apomicts, such a method relies on pooling genomic DNA subsets from progeny plants showing extreme classes for the mode of reproduction, and then screening for molecular polymorphisms between apomictic and sexual individuals using DNA markers. This approach enables the analysis of a large number of genomic traits and increases the reliability of polymorphisms linked to apomixis and its components (Labombarda et al. 2002; Vijverberg et al. 2004).

The experimental evidence for simple inheritance of apomixis components is supported by a number of genetic mapping studies using molecular markers in both aposporic and diplosporic species. Mapping results have confirmed the simple, dominant inheritance of apomixis components, corresponding to one chromosomal region or a few chromosomal blocks (Table 20.1). With the exception of

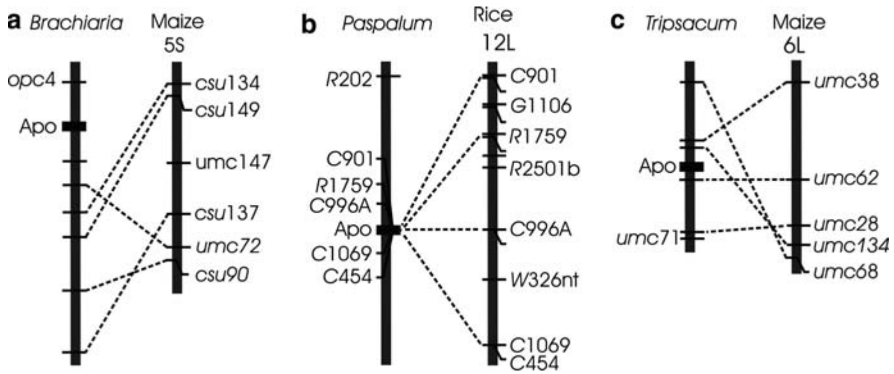


Fig. 20.3 Syntheny between *Brachiaria* (**a**), *Tripsacum* (**c**) and maize: the apomixis locus (Apo) has been mapped using heterologous probes and/or primers. **b** Alignment of chromosome regions carrying the Apo locus in *Paspalum simplex* with its homologue of rice localized on the telomeric region of the long arm of chromosome 12

T. officinale (van Dijk et al. 2003), strong suppression of recombination around the loci linked to apomeiosis has been found in all documented cases. In particular, the association of apospory and diplospory with chromosomal regions showing suppressed recombination has now been observed in aposporic (*P. squamulatum* and *P. simplex*) and diplosporic (*E. annuus* and *T. dactyloides*) species (reviewed in Ozias-Akins 2006; Vijverberg and van Dijk 2007). Surprisingly, the pattern of low recombination has been "broken" upon construction of a saturated molecular linkage map of the diplospory region in *Taraxacum* (Vijverberg et al. 2004). A bulked segregant analysis was used to identify and map molecular markers in the region harbouring the diplospory locus and spanning a length of about 20 cM, although none was found to be strictly linked with diplospory. This species provides a unique case of genetic recombination in a chromosomal region carrying genes for apomeiotic embryo sac formation. Molecular markers linked with parthenogenesis have been identified in *P. pratensis* (Barcaccia et al. 1998) and *E. annuus* (Noyes and Rieseberg 2000), although no evidence for suppression of genetic recombination was found in the corresponding chromosomal regions (Table 20.1).

An important contribution to the mapping of the apomixis trait was given by synteny between specific chromosomal regions in apomicts and sexual crops and by the exploitation of heterologous probes. As far as apospory is concerned, genetic mapping studies were performed in *Brachiaria brizantha* (Pessino et al. 1997, 1998), *Paspalum simplex* (Pupilli et al. 2001, 2004; Labombarda et al. 2002), *P. squamulatum* (Ozias-Akins et al. 1998) and *P. pratensis* (Barcaccia et al. 1998). In *B. brizantha*, an intrageneric cross at the tetraploid level ($2n=4x=36$) was investigated mainly using RFLP markers with heterologous maize probes related to the short arm of chromosome 5. The apomixis locus was mapped in a genetic window longer than ~20 cM, with six markers all positioned at one of the flanking sides (Fig. 20.3a). Two additional AFLP markers co-segregating with apomixis were mapped at 1.2 and 5.7 cM from the apomixis locus, spanning the

trait in a total length of 6.9 cM (Pessino et al. 1998). In *P. simplex*, a progeny segregating for apomixis was obtained by backcrossing an intraspecific tetraploid hybrid ($2n$=$4x$=40). Five RFLP markers detected by using heterologous rice probes, spanning a 15-cM region in the long arm of chromosome 12, were mapped at 0 cM from the putative apomixis locus (Fig. 20.3b). Four additional AFLP markers were found tightly clustered at this locus, one of which tested to be hemizygous, being present only in the apomicts (Labombarda et al. 2002). A comparative mapping analysis revealed the apomictic chromosomal regions in *P. simplex* to be largely and partly conserved in the close relatives of *P. melacophyllum* and *P. notatum* respectively (Pupilli et al. 2004). This result suggested that a relatively small proportion of the chromosome carrying the apomixis locus is structurally and functionally conserved in *Paspalum*. In *P. squamulatum*, 12 random amplified polymorphic DNA markers were found strictly co-segregating with apospory (Ozias-Akins et al. 1998). Of the six low-copy number DNA clones derived from these markers, four appeared to be hemizygous, and all were found to be conserved in apomictic individuals of the close relative *Cenchrus ciliaris* (Roche et al. 1999). Subsequent FISH experiments with the apospory-related BACs (bacterial artificial clones) supported the conservation of the apospory-specific genomic region in *P. squamulatum* and *C. ciliaris* (Roche et al. 2002) and revealed the localization of this region on the short arm of a single chromosome (Goel et al. 2003). Sequencing work revealed a high abundance of repetitive elements from a retrotransposon family in the apospory-specific chromosomal region (Akiyama et al. 2004). Parthenogenesis has been mapped in *P. pratensis* only, with nine AFLP markers equally distributed on both sides of the putative locus, being the closest markers found at 6.6 and 8.8 cM (Barcaccia et al. 1998; Albertini et al. 2001b).

For diplospory, genetic mapping studies were performed in *T. dactyloides* ($2n$=$4x$=72) using a segregating population from an intergeneric cross with *Z. mays* ($2n$=$2x$=20; Grimanelli et al. 1998), in *E. annuus* ($2n$=$3x$=27) using a segregating population from an interspecific cross with *E. strigosus* ($2n$=$2x$=18; Noyes and Rieseberg 2000) and in *T. officinale* using a segregating population from an intraspecific cross between diploid sexual and tetraploid aposporic plants of common dandelion (van Dijk and Bakx-Schotman 2004; Vijverberg et al. 2004). In *Tripsacum*, three RFLP markers detected by means of heterologous maize probes, spanning a ~40 cM region in the long arm of chromosome 6, were found to be strictly inherited with the apomixis trait (Fig. 20.3c), although they showed recombination on the corresponding maize homologues (Grimanelli et al. 2001b). In *Erigeron*, as many as 11 AFLP markers closely co-segregated with diplospory and one additional marker was mapped 2 cM apart from the target locus, while four AFLP markers co-segregated with parthenogenesis and spanned a 20-cM distance on a different linkage group (Noyes and Rieseberg 2000). In *Taraxacum*, a linkage group showing a total length of 18.6 cM was constructed with markers found on both sides of the diplospory locus in regions 5.9 and 12.7 cM long (Vijverberg et al. 2004). No AFLP markers fully co-segregated with diplospory, and the closest AFLP markers were located at 1.4 cM on both flanking sides. Several additional AFLP markers were later mapped in the same region using a larger segregating

population. The results were consistent with the lack of suppressed recombination in the chromosomal region surrounding the diplospory locus (Vijverberg and van Dijk 2007).

Further cytogenetic characterization of apomixis chromosomal regions was carried out in a few species such as *P. squamulatum* (Ozias-Akins et al. 1998), *P. simplex* (Labombarda et al. 2002) and *T. officinale* (Vijverberg and van Dijk 2007), by performing FISH experiments using apomixis-associated BACs. With the exception of *Taraxacum*, overall results confirmed the existence of a strong suppression of genetic recombination in all species, supporting physical lengths of ∼50 Mbp (Akiyama et al. 2004) up to ∼100 Mbp (Calderini et al. 2006). This suppression of recombination has hindered subsequent high-resolution genetic mapping and map-based cloning strategies aimed at isolating the genes for apomixis in these species.

In conclusion, genomic loci for apomixis as a whole or apomeiosis alone are defined by large chromosomal regions in most species, suggesting the presence of several linked genes, rather than a single one. The strong suppression of genetic recombination found in most apomixis chromosomal regions mapped so far by means of molecular markers demonstrates both diversification of allele sequences at these loci, compared to homologous regions in sexual relatives, and violation of synteny between apomictically reproducing species and phylogenetically correlated sexual species, e.g. *P. squamulatum*, *P. simplex* and *T. dactyloides*. In fact, those species which lacked evidence for strong suppression of genetic recombination in the apomixis chromosomal regions, i.e. *T. officinale* for diplospory, *B. bryzantha* for apospory, and *E. annuus* and *P. pratensis* for parthenogenesis, were characterized by the independent inheritance of apomeiosis and parthenogenesis. This finding indicates that, in species or genera where apomicts and close sexual relatives still exist, genetic divergence between apomictic and sexual forms is limited. By implication, relationships between parental lines and types of crosses can influence the success of genetic linkage mapping studies and apomixis gene cloning strategies.

Attempts to introgress apomixis from natural apomicts into crop species have failed (Spillane et al. 2001), and efforts to identify apomixis genes in natural apomicts by map-based cloning have been hampered by the finding that apomixis is associated with large genomic regions which are repressed for recombination (Grimanelli et al. 1998; Ozias-Akins et al. 1998; Pessino et al. 1998; Pupilli et al. 2004).

20.7 Advanced Biotechnological Approaches: Looking for Candidate Genes and Engineering Apomixis

Although many years of descriptive studies have provided a solid documentation of the types of apomictic processes occurring in a wide variety of plant species, molecular studies aimed at understanding the basis of apomixis have shed little

information on its central mystery, partly because the majority of apomicts do not constitute agriculturally important crops and, with few exceptions (e.g. *Tripsacum* and maize), do not have agriculturally important relatives (Bicknell and Koltunow 2004; Albertini et al. 2005). Zygotic embryogenesis (sexuality) and apomeiotic parthenogenesis (apomixis) are thought to follow similar pathways during embryo and seed production. Specific genes are activated, modulated or silenced in the primary steps of plant reproduction to ensure that functioning embryo sacs develop from meiotic spores and/or apomeiotic cells. As additional genes may be specifically or differentially expressed in sexually and apomictically reproducing plants, and operate during embryo development, we would be better equipped to understand apomixis if the genes responsible for controlling the specific and differential expression in embryo sac and embryo formation were to be detected. Chaudhury and Peacock (1993) hypothesized that genes isolated in model species, such as Arabidopsis (*Arabidopsis thaliana*), would be important for the study of apomixis. The advantages of such a strategy reside in (1) the possibility of using, in apomictic species, molecular tools developed in Arabidopsis and other model species, as associated with the reproduction system and (2) trying to understand the function of genes putatively involved in apomixis by studying these in the model sexual species. For this reason, advanced research on apomixis is generally divided into two complementary approaches: (1) analysis of the trait in natural apomictic species and (2) functional analysis of genes involved in female sporogenesis and seed development in species which normally form seeds by sexual reproduction.

Mutagenesis approaches aimed at identifying genes deregulating steps which are fundamental in circumventing meiotic reduction (apomeiosis), in activating embryo development without fertilization (parthenogenesis), and in initiating and maintaining the formation of functional endosperm have served to isolate mutants with apomictic characteristics in *Arabidopsis* and other model species by loss-of-function mutagenesis screenings. These approaches are generally based on either allowing fertilization and then screening for purely maternal inheritance in the progeny, or preventing fertilization and isolating pseudo-suppressors which allow seed development to take place in the absence of fertilization (Curtis and Grossniklaus 2007). Of these, the screens for fertilization-independent seed development have led to the identification of mutants now known as the fertilization independent seed (*fis*) class mutants (Grossniklaus et al. 2001b), all of which are able to initiate endosperm development in the absence of fertilization. In particular, proteins codified by three of these genes, FIS1 (or MEA), FIS2 and FIS3 (or FIE), repress cell proliferation in the central cell in sexual plants in the absence of fertilization (Ohad et al. 1996; Grossniklaus and Schneitz 1998; Kiyosue et al. 1999; Luo et al. 1999; Vielle-Calzada et al. 1999; Grossniklaus et al. 2001b; Lohe and Chaudhury 2002; Hsieh et al. 2003; Guitton and Berger 2005). This suggests that a number of developmental checkpoints must be deregulated in the sexual process before viable seed is generated in the absence of fertilization (Curtis and Grossniklaus 2007).

Nowack et al. (2007) demonstrated that it is possible to obtain viable fertilized seeds with uniparental diploid endosperm of maternal origin when the maternal FIS machinery is impaired. It has also been demonstrated that loss of function of

MET1, leading to hypomethylation of the maternal gametophyte in *fie*1 mutants, gives rise to an endosperm formation very similar to that associated with sexual reproduction. Other proteins interacting with MEA (FIS), such as the origin recognition complex (ORC), might also play a role in the apomictic mode of reproduction. Another gene, identified with a loss-of-function approach, is *Multicopy Suppressor of Ira1* (*MSI1*). Guitton and Berger (2005) demonstrated that *msi* mutants are characterized by spontaneous division of the egg cell, even though parthenogenetically derived embryos aborted early in development and did not form viable seeds.

An alternative approach is to generate synthetic apomictic traits using the gain-of-function approach, which seems very promising because the genes controlling elements of apomixis behave as dominant factors in crosses with sexual relatives (Savidan 2000; Grimanelli et al. 2001a; Grossniklaus et al. 2001a, Richards 2003; Curtis and Grossniklaus 2007). One of the simplest strategies is to place a candidate gene under the transcriptional control of a heterologous promoter (Curtis and Grossniklaus 2007), but the identification of candidate genes in sexually reproducing plants has been a difficult task. In fact, it has resulted in the isolation of only a small number of genes involved in the acquisition of embryogenic competence from somatic cells, e.g. somatic embryogenesis receptor-like kinase (*SERK*; Schmidt et al. 1997; Hecht et al. 2001), and spontaneous induction of embryo production when overexpressed (*LEC*1, *LEC*2) or repressed (*PKL*; Ogas et al. 1999). An activation tagging approach was used by Zuo et al. (2002) to identify genes of which the overexpression could induce the formation of somatic embryos in *Arabidopsis* tissues without the need for external hormonal treatments. This resulted in the isolation of an allele, PGA6, which was found to be identical to WUSCHEL (WUS), a homeodomain protein previously shown to be involved in specifying stem cell fate in shoot and floral meristems. WUS PGA6 presumably promotes a vegetative-to-embryogenic transition and/or maintains embryonic stem cell identity (Fehér et al. 2003). Another candidate was identified by induction in microspore cultures of *Brassica napus* undergoing somatic embryogenesis (Boutilier et al. 2002). The gene was named *babyboom* (*bbm*) because, when overexpressed under the control of the 35S promoter, it led to the ectopic formation of embryos and cotyledons on leaves. Genes sharing similarity with BBM have been isolated in several other species but maybe the most important finding is the isolation of the *ASGR-BBM* in the apospory-specific genomic region (ASGR) of *P. squamulatum* (Conner et al. 2007). The *ASGR-BBM* transcript encodes a 545-amino acid protein containing two AP2 domains which are 96% similar to the AP2 regions of *BnBBM*. Outside of the AP2 domains, similarity of *ASGR-BBM* to *BnBBM* declines significantly (35% similarity upstream and 27% similarity downstream; Conner et al. 2007).

Studies have also been performed on other species carrying mutations resembling components of apomixis. For example, two genes classified as MOB1-like have been identified in an apomeiotic mutant of *Medicago sativa* (*TWO-N-EGG*; Citterio et al. 2005) as being involved in cell proliferation and programmed cell death within reproductive organs. It has been demonstrated that, in addition to

alfalfa, other plant genomes—e.g. the sexual diploids Arabidopsis and rice, and the apomictic polyploids *P. pratensis* and *Hypericum* spp.—contain MOB1-related genes (Barcaccia et al. 2001; Citterio et al. 2005).

Diploid cells in place of normal haploid megaspores have been observed recently in Arabidopsis, resulting from mutations of the *SWI*1 (*SWITCH*1/*DYAD*) gene (Ravi et al. 2008). The occurrence of apomeiosis by mutation of a single gene coding for a phospholipase C which controls sister chromatid cohesion and centromere organization during sporogenesis was demonstrated. These findings represent a significant step towards the synthesis of apomixis by the manipulation of genes which function in normal sexual development.

The second main approach requires searching for candidate apomixis genes in species where the trait occurs naturally. For this reason, transcriptional profiling procedures have been proposed to compare transcripts of sexual and apomictic reproductive cell types, but these are always hampered by the low accessibility of the female gametophyte and by the high ploidy level of apomictic species. Molecular differential screening of plants with contrasting modes of reproduction is one of the most powerful tools which can be applied to identifying, mapping and isolating the gene(s) putatively involved in apomixis. Many new techniques have been designed in recent years (Green et al. 2001). All assess new genes but while some focus on obtaining expression data and high-throughput data, others aim at identifying new and rare, differentially expressed transcripts. Some require large amounts of material to be analyzed and pre-existing genomic knowledge. One of the new techniques is based on microarrays (Brown and Botstein 1999), which allows a genome-wide expression profile of thousands of genes to be performed in one experiment. Though powerful, this approach is expensive and can be readily applied only to model species for which significant genomic information is available (Baldwin et al. 1999). Unfortunately, genetic annotation in higher eukaryotes is limited to a few models and information on less well-characterized species is poor, and likely to remain so for some time. Moreover, because rarely expressed transcripts are usually missing from cDNA libraries due to overrepresentation of abundant messengers, microarrays could fail to detect genes which are rare but fundamental for traits like apomixis. Differential display (DD), PCR-derived techniques which share gel separation and visualization procedures, but differ in the methods adopted for generating amplified cDNA fragments, would be more suitable for identifying low-expressed genes (Reijans et al. 2003). mRNA fingerprinting strategies permit a large number of fragments to be analyzed, and increase the reliability of differentially expressed transcript detection which starts from very small amounts of messengers (Bachem et al. 1996). This feature is essential when DD is applied to tissues where it is hard to isolate stage-specific mRNAs, such as small florets. cDNA-AFLP (Bachem et al. 1996) has proved the most popular procedure because of its ability to detect differentially expressed genes. It has good reliability and sensitivity, and correlates well with Northern analysis (Durrant et al. 2000; Jones et al. 2000; Barcaccia et al. 2001; Donson et al. 2002; Cnudde et al. 2003). The reproducibility is very high compared to that of microarray and GeneChip technologies (Reijans et al. 2003). A possible drawback of the technique

is that more than one band is expected to be visualized for each transcript (Matz and Lukyanov 1998). However, redundancy can be very informative in cases of alternative splicing.

Comparative gene expression studies have been carried out during the early stages of apomictic and sexual embryo sac development in *Panicum maximum* (Chen et al. 1999), *Brachiaria* species (Leblanc et al. 1997; Rodrigues et al. 2003), *Pennisetum* (Vielle-Calzada et al. 1996; Jessup et al. 2003) and *Paspalum* (Pessino et al. 2001). However, most of these were based on subtractive hybridization techniques and isolated only a few genes to which, disappointingly, no clear function could be assigned. Hybridization-based studies, even if negative in context, add support to the proposal that sexual and apomictic developmental pathways differ primarily in their ability to regulate common elements (Bicknell and Koltunow 2004). In support of this hypothesis, Tucker et al. (2003) and Albertini et al. (2004) have demonstrated that the developmental program is highly conserved during zygotic embryogenesis and apomeiotic parthenogenesis and, on the basis of available results, natural apomixis does not seem to result from the failure of a single gene of the reproductive pathway, but rather from epistatic, possibly silencing action, exerted on the normal sexual reproduction pathway by a set of genes inherited as a unit and evolved in polyploid plants (Ozias-Akins et al. 1998).

Laspina et al. (2007) carried out a full-transcriptome survey in order to isolate genes differentially expressed in immature inflorescences of sexual and aposporous *P. notatum* genotypes. Differential display experiments were used to check the expression of about 10,000 transcripts, and led to the identification of 71 unigenes expressed either in the aposporous or in the sexual genotype, whereas functional annotation was achieved for 39 of them.

Perhaps the most thorough study using a transcript profiling approach comes from *P. pratensis* where cDNA-AFLP analysis resulted in the isolation of fragments which were specific to carefully staged florets of either a sexual or apomictic genotype and were not present in leaves (Albertini et al. 2004). Most of the cDNA sequences were not specifically expressed in apomictic or sexual genotypes, but rather their expression was differentially modulated or quantitatively different (Albertini et al. 2004, 2005), lending additional support to the hypothesis that apomixis results from a deregulated sexual pathway (reviewed in Ozias-Akins 2006). In particular, PpSERK and APOSTART were characterized (Albertini et al. 2005), and they seem to be involved in cell-to-cell interaction for both the signalling pathway and hormone stimulation. These authors proposed that PpSERK gene activation in nucellar cells of apomictic genotypes is the switch which channels embryo sac development and that it could redirect signalling gene products to compartments other than their typical ones. The SERK-mediated signalling pathway may interact with the auxin/hormonal pathway controlled by APOSTART. Moreover, since BLAST analysis of sequences revealed that homologies of APOSTART, PpSERK, PpMET, PpARM and other genes are tightly linked in a small chromosome region of Arabidopsis, *M. truncatula* and rice, attempts were made using the physical mapping in *P. pratensis* to determine whether the linkage was maintained. Preliminary results indicate a strong co-segregation of clones carrying

PpSERK, APOSTART and other genes such as PpMET (Albertini et al. 2007). In addition, partial/complete cDNA fragments showing homology to APOSTART have also been isolated from *P. squamulatum*, *C. ciliaris* and *H. perforatum* by other research groups, and spatial/temporal characterization studies are in progress. In fact, if these genes are truly involved in apomixis, then irrespective of the species under study, they should conserve their involvement in this modification of the reproductive system.

In *H. perforatum*, a transcription profile approach of sporogenesis and gametogenesis performed by mRNA profiling of unripened anthers and unpollinated pistils led to the isolation of several transcripts specifically expressed in pistils of the highly apomictic ecotype, including an EST showing similarity to a gene coding for an ATPase RNA helicase responsible for an embryo defective phenotype in *Arabidopsis* (MEE29, i.e. maternal effect embryo). This gene, termed *HpMEE29-like*, was found to be differentially expressed between aposporic and meiotic plants of *H. perforatum* (Galla and Barcaccia, unpublished data). Moreover, a RING-finger gene (i.e. *HpARIADNE*), the DNA markers of which were found to be in strong linkage disequilibrium with the apomixis trait, is also under study in *H. perforatum* (Barcaccia et al. 2007).

More recently, the apomixis research group of IPK has applied a high-throughput differential display approach (SuperSAGE) to study naturally occurring quantitative variations in gene expression between ovules of apomictic and sexual *Boechera holboellii* genotypes (Sharbel et al. 2009). Using SuperSAGE, they have identified over 6,000 differentially expressed mRNA tags in ten microdissected ovules from two sexual and two diploid apomictic accessions. The genes to which the mRNA tags belong were determined by homology searches to sexual and apomictic flower-specific transcriptome libraries which were sequenced using 454 technology. Comparisons between the sexual and apomictic ovules show that many of the differentially expressed mRNAs are of low copy number. Nevertheless, both allele-specific expression and microduplication can explain the observed variation between reproductive modes. The use of deep transcriptomic analyses of living microdissected tissue, in conjunction with massively parallel transcriptome sequencing, has thus enabled the identification of a large set of candidate alleles which will be the subject of subsequent analyses of expression profiles at different developmental stages and in different genetic backgrounds.

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Chapter 21
Male Sterility

C.D. Chase, A. Ribarits, and E. Heberle-Bors

21.1 Introduction

The control of pollen fertility is central to the production of F1-hybrid seed in self-pollinating crops, and is potentially applicable to the containment of transgenes deployed in crop plants. Pollen sterility can be achieved through cytoplasmic male sterility (CMS) encoded by the plant mitochondrial genome, or through genic male sterility encoded by the nuclear genome. Both routes have been exploited in schemes for hybrid seed production. Recently, pollen sterility has been achieved through novel strategies involving nuclear or plastid transgenes. Here we review the applications of pollen sterility, and the genetic systems used for the control of pollen fertility.

21.2 Applications of Pollen Sterility

While F1-hybrid seed production has been the primary driving force behind strategies to achieve pollen sterility, the control of pollen function also plays a role in other valued phenotypes. Furthermore, the expanding plantings of transgenic crops have created interest in the use of pollen sterility to limit the flow of transgenes to nearby native plants and non-transgenic crops.

C.D. Chase
Horticultural Sciences Department, University of Florida, Gainesville 32611 FL, USA
e-mail: cdchase@ufl.edu

A. Ribarits and E. Heberle-Bors
Department of Plant Molecular Biology, Max F. Perutz Laboratories, 1030, Vienna Austria

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21.2.1 Hybrid Seed Production

Hybrid vigor (or heterosis), uniformity, broad adaptation, and variety protection are desired features of crop plants that can be achieved through the use of F1-hybrid varieties (Duvick 1959, 2001; Perez-Prat and van Lookeren Campagne 2002; Virmani et al. 2003; Troyer 2006; Springer and Stupar 2007; Cheng et al. 2007). In the case of self-pollinating crops, uniform populations of pollen-sterile plants must be created, to then be cross pollinated for the production of F1-hybrid seed.

21.2.1.1 Hybrid Seeds via Cytoplasmic Male Sterility

In hybrid seed production, pollen sterility is often achieved through the use of CMS (Duvick 1959; Budar and Pelletier 2001; Havey 2004; Pelletier and Budar 2007; Cheng et al. 2007). Maternally inherited CMS results from genes in the plant mitochondrial genome (reviewed in Budar and Pelletier 2001; Hanson and Bentolila 2004; Chase 2007). These genes do not segregate, but expression of the CMS trait can be conveniently controlled by the presence or absence of nuclear restorer-of-fertility (restorer) alleles (reviewed in Schnable and Wise 1998; Wise and Pring 2002; Hanson and Bentolila 2004; Chase 2007; Pelletier and Budar 2007). Uniform populations of pollen-sterile plants are developed by crossing CMS plants with normal-cytoplasm, pollen-fertile plants that do not carry nuclear restorer alleles. The resulting pollen-sterile progeny produce F1-hybrid seed via cross fertilization with pollen-fertile plants grown nearby. Pollen fertility may or may not be restored in the F1 generation. If the F1 seed is grown to produce vegetative structures such as roots, tubers, or leaves, pollen fertility need not be restored in the F1 generation (Duvick 1959; Havey 2004). In the case of hybrid crops grown for a seed product, pollen fertility is achieved in the F1 generation via nuclear restorer alleles introduced in the previous generation (Duvick 1959; Havey 2004). Alternatively, pollen-sterile F1 hybrids can be grown along with male-fertile pollinators. In crops such as maize, F1-hybrid seed can be produced through the use of CMS or through hand emasculation (Duvick 1959). CMS (pollen-sterile) and normal-cytoplasm (pollen-fertile) versions of the same hybrid can be produced and blended for planting (Stamp et al. 2000; Weingartner et al. 2002a, b; Havey 2004).

In some cases, CMS confers a yield advantage independent of heterosis. Pollen-sterile CMS maize hybrids, fertilized with isonuclear pollen-fertile hybrids, yield more grain than do isonuclear pollen-fertile hybrids (Stamp et al. 2000). The Plus-Hybrid system combines CMS and a xenia effect to create a further yield advantage when pollen-sterile CMS hybrids are crossed with unrelated pollinators (Weingartner et al. 2002a, b). CMS lines of wheat out-yield isonuclear lines produced through use of chemical sterilizing agents (Adugna et al. 2004). Work in sorghum (Moran and Rooney 2003) and millet (Chandra-Shekara et al. 2007)

demonstrates genetic variation of male sterility-inducing cytoplasms with respect to yield.

CMS is used for hybrid seed production in many crops, including alfalfa, onion, sugar and table beets, oilseed and vegetable brassicas, maize, millet, rice, pepper, rye, sorghum, sunflower, and wheat (Duvick 1959; Havey 2004; Adugna et al. 2004; Cheng et al. 2007). T-cytoplasm maize carries an unusual CMS gene that also conditions susceptibility to the fungal pathogen *Bipolaris maydis* (reviewed in Pring and Lonsdale 1989; Wise et al. 1999). Although this caused concerns regarding the use of CMS for crop production, CMS genes are diverse and work by different mechanisms (Schnable and Wise 1998; Hanson and Bentolila 2004; Linke and Borner 2005; Chase 2007; Carlsson et al. 2008). Hence, undesirable phenotypes are not necessarily expected to result from every application of CMS in hybrid crop production.

21.2.1.2 Hybrid Seeds via Nuclear Male Sterility

In contrast to CMS, pollen sterility conditioned by dominant or recessive nuclear mutations is not true-breeding. The male-sterile plants must be propagated through pollination with wild-type, male-fertile plants. In some cases, this problem can be circumvented. Genic male-sterile leek plants are propagated asexually, and then cross pollinated to produce hybrid seed (Smith and Crowther 1995; Havey 2004). In China, considerable hybrid rice seed is developed through environmental genic male sterility systems. In photo-thermo genic male sterility, the seed parent line is amplified under environmental conditions that cause this line to be pollen-fertile (cool temperatures and shorter photoperiod). For the production of F1-hybrid seed, the seed parent is grown and cross-fertilized under environmental conditions that cause pollen sterility (warmer temperatures and longer day-length; Virmani et al. 2003; Cheng et al. 2007). Female parent propagation has been described as the key limiting factor for the production of hybrids in many crops (Perez-Prat and van Lookeren Campagne 2002). Asexual propagation is labor-intensive, and environmental factors are not always uniform enough to ensure complete pollen sterility. Novel strategies to address these challenges via the deployment of nuclear transgenes are described below.

21.2.2 *Value-Added Traits*

The control of pollen fertility has implications for plant improvement beyond hybrid seed production. Pollen sterility, combined with parthenocarpy, results in the production of desirable seedless fruits (Possingham 1998; Schijlen et al. 2007). Pollen is an allergen of significant importance (Goldberg et al. 1998; Mahillon et al. 2006), and pollination induces the rapid senescence of floral organs in many species

(Rubinstein 2000; Rogers 2006). Hence, ornamental species might be improved through control of pollen fertility (Daniell 2002).

21.2.3 Transgene Containment

The expansion of transgenic crops has raised concerns about the flow of transgenes to nearby cross-compatible crops and native plants (Daniell 2002; Chapman and Burke 2006; Lee and Natesan 2006; Brunner et al. 2007). Many factors influence transgene flow, including dispersal via seeds, pollen, or vegetative propagules (Brunner et al. 2007), and the fitness of the plants resulting from genetic exchange with the transgenic crop (Chapman and Burke 2006; Lee and Natesan 2006). No single strategy of containment will be 100% effective in any situation, and the best combined approaches must be determined on a case-by-case basis (Chapman and Burke 2006; Lee and Natesan 2006). Pollen sterility can make a significant contribution toward transgene containment (Brunner et al. 2007). This strategy is suited to species amenable to vegetative propagation, and has been applied to creeping bentgrass (Luo et al. 2005). The maize Plus-Hybrid system can also be adapted to limit transgene flow, through use of a pollen-sterile transgenic hybrid and a non-transgenic pollinator (Feil et al. 2003). Transgene containment in F1 hybrids of seed crops is also possible by crossing a homozygous male-sterile tobacco line, produced by metabolic engineering of glutamine, with a homozygous male-fertile line. The resulting heterozygous F1 plants produce 50% fertile pollen, and all of the fertile pollen is non-transgenic (Ribarits et al. 2007; see below).

21.3 Cytoplasmic Male Sterility Systems

CMS is observed in a wide array of plant species that exhibit diversity with respect to the causal gene and its phenotypic effects. Although the maternally inherited mitochondrial genome encodes CMS, nuclear restorer alleles regulate the expression of this trait (Schnable and Wise 1998; Wise and Pring 2002; Hanson and Bentolila 2004; Chase 2007).

21.3.1 CMS Genes

Most CMS genes are mosaic open reading frames (orfs) that include segments from normal mitochondrial gene coding and flanking sequences, and segments of unknown origin (Schnable and Wise 1998; Hanson and Bentolila 2004; Linke and

Borner 2005; Kubo and Newton 2008). These orfs, when fused to plant mitochondrial promoters or co-transcribed courtesy of upstream mitochondrial genes (Hanson and Bentolila 2004), become gain-of-function mutations. Recombination in plant mitochondrial genomes likely favors the creation of CMS genes, and places these in contexts that support gene expression (Sandhu et al. 2007; Kubo and Newton 2008). Functional copies of all essential mitochondrial genes are almost always retained in CMS mutants. Two examples of CMS involving essential mitochondrial respiratory genes, deletion of the NADH dehydrogenase subunit 7 gene in *Nicotiana sylvestris* (Pla et al. 2005), and truncation of the cytochrome oxidase subunit 2 gene in wild beet (Ducos et al. 2001), seem not to condition deleterious vegetative phenotypes. Perhaps alternative pathways of electron flow in plant mitochondria (Rasmusson et al. 2004; Rhoades and Subbaiah 2007; Noctor et al. 2007) compensate for the effects of these mutations.

21.3.2 CMS Phenotypes

CMS genes condition a wide array of reproductive abnormalities (Laser and Lersten 1972; Schnable and Wise 1998; Zubko 2004; Hanson and Bentolila 2004; Linke and Borner 2005; Chase 2007; Carlsson et al. 2008). In some cases, male reproductive organs (stamens) are transformed into petals or into female reproductive organs (carpels). Other CMS mutations condition degeneration of anthers and/or developing pollen.

21.3.2.1 Homeotic CMS

CMS involving the transformation of stamens to petals (petaloid) or stamens to carpels (carpeloid; Zubko 2004; Linke and Borner 2005; Carlsson et al. 2008) copies the phenotypes conditioned by mutations in nuclear floral organ identity genes (Coen and Meyerowitz 1991; Krizek and Fletcher 2005). In dicotyledonous plants, four whorls of plant floral organs (sepals, petals, stamens, and carpels) develop in response to spatial patterns established by three major classes of floral organ identity genes (A, B, and C). These encode MADS box transcription factors having distinct domains of expression that overlap, in part, to specify sepals (A alone), petals (A and B), stamens (B and C), and carpels (C alone). Decreased accumulation of B-class MADS box transcription factors occurs in several carpeloid CMS systems (Murai et al. 2002; Linke et al. 2003; Zubko 2004; Teixeira et al. 2005). Unusually, late expression of B-class genes is associated with abnormal anther and pollen development of CMS *B. napus* of the Napus (*nap*) type (Geddy et al. 2005). Nuclear MADs box genes are therefore targets of mitochondrial influence. Further study of homeotic CMS will enhance our understanding of retrograde (mitochondria-to-nucleus) signaling pathways in plants.

21.3.2.2 Degenerative CMS

In CMS sunflower, anther tissues exhibit features of apoptotic programmed cell death (PCD) observed in vertebrates, namely, the release of cytochrome *c* from the mitochondria, cleavage of nuclear DNA, and condensation of cytoplasm containing intact organelles (Balk and Leaver 2001). Declining ATP levels promote PCD in animal cells (Bras et al. 2005), and perhaps in sunflower, where the CMS gene encodes a protein related to mitochondrial ATP synthase subunit 8. Abundance of the ATP synthase complex is reduced in CMS compared to male-fertile sunflower (Sabar et al. 2003). The identity and targets of mitochondrial cell death signals in plants are not yet clear, as the caspase proteases, primary downstream target of mitochondria-signaled PCD in animals, are not conserved in plants (Jones 2000; Lam 2004; Logan 2006).

When ATP levels are insufficient to support PCD, cell death can occur by necrosis (Bras et al. 2005). In CMS-T maize plants, the tapetal cells lining the anther exhibit features of necrotic cell death, including the swelling and lysis of organelles and cells (Warmke and Lee 1977). In vegetative tissues, the CMS-T gene product (URF13) forms a mitochondrial pore upon exposure to a toxin produced by *Bipolaris maydis* (reviewed in Wise et al. 1999). In tapetal cells, this pore might form in the absence of toxin, leading to mitochondrial dysfunction and necrosis (Flavell 1974).

21.3.2.3 Male Specificity

In many cases, CMS gene products accumulate throughout the plant, but condition a phenotype only in male reproductive organs (Dewey et al. 1987; Wise et al. 1987; Nivison and Hanson 1989; Laver et al. 1991; Krishnasamy and Makaroff 1994). Warmke and Lee (1978) observed that a 20- to 40-fold increase in the number of mitochondria accompanies the normal development of tapetal cells and pollen within the anther. They proposed that CMS mutations compromise the energy status of tapetum and/or developing pollen. Energy considerations might explain both homeotic and degenerative CMS phenotypes, if the proper regulation of nuclear floral organ identity genes requires ATP-dependent proteolysis (Teixeira et al. 2005), and if declining levels of ATP trigger mitochondria-signaled PCD in anther tissues (Sabar et al. 2003). Alternatively, positive regulators of cell death produced by anthers or developing pollen (Flavell 1974) might induce or act synergistically with mitochondrial cell death signals, or negative regulators might repress these pathways in all but male reproductive organs. The large number of mitochondria in male reproductive organs (Warmke and Lee 1978) could amplify such mitochondrial signaling pathways.

21.3.3 Fertility Restoration

Expression of CMS can be suppressed by system-specific nuclear restorer alleles. The molecular cloning of several restorer genes identified pentatricopeptide repeat (PPR) protein coding genes in all but one case (reviewed in Schanble and Wise 1998; Wise and Pring 2002; Hanson and Bentolila 2004; Chase 2007).

21.3.3.1 Fertility Restoration Genetics

Nuclear fertility restoration systems can be sporophytic, acting in the diploid plant, or gametophytic, acting in the haploid pollen. In sporophytic restoration, all pollen produced by a CMS plant heterozygous for a dominant restoring allele (CMS *Rf*/*rf*) will function regardless of pollen genotype. In gametophytic restoration, only pollen carrying the restoring allele will function. Some fertility restoration systems require the complementary action of restoring alleles at two loci (Pring et al. 1999; Wise et al. 1999), and some CMS systems can be reversed through the action of two or more independent restoration systems (Zabala et al. 1997; Sarria et al. 1999; Wang et al. 2006).

21.3.3.2 Fertility Restoration Mechanisms

In many cases, mitochondria-encoded CMS gene products fail to accumulate in the presence of a nuclear restorer allele (Dewey et al. 1987; Nivison and Hanson 1989; Krishnasamy and Makaroff 1994; Moneger et al. 1994; Abad et al. 1995; Wang et al. 2006). The decreased abundance or internal cleavage of CMS transcripts accompanies this protein loss in some (Dewey et al. 1987; Pruitt and Hanson 1991; Moneger et al. 1994; Wang et al. 2006) but not all cases (Chase 1994; Krishnasamy and Makaroff 1994). It is not known whether these transcript effects are the basis for the protein phenotype, or a secondary consequence of failure to translate a CMS transcript.

21.3.3.3 Fertility Restoration Genes

Rf2 of CMS-T maize encodes a mitochondrial aldehyde dehydrogenase (Cui et al. 1996; Liu et al. 2001), and restores pollen fertility through a mechanism of metabolic compensation. All other restorer genes cloned to date encode PPR proteins (Bentolila et al. 2002; Kazama and Toriyama 2003; Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003; Akagi et al. 2004; Komori et al. 2004; Klein et al. 2005; Wang et al. 2006), members of a highly expanded plant protein family (Lurin et al. 2004). PPR proteins are comprised largely of degenerate

35-amino acid repeats, which might confer sequence-specific RNA binding capability to mediate interactions between enzymes and RNA substrates (Lurin et al. 2004; Stern et al. 2004; Shikanai 2006; Delannoy et al. 2007). In Arabidopsis, 54% of the PPR proteins are predicted to be located in the mitochondria, and 19% are predicted to locate in the plastids (Lurin et al. 2004). PPR proteins function in the translation (Schmitz-Linneweber et al. 2005), editing (Kotera et al. 2005; Okuda et al. 2007), processing and splicing (Schmitz-Linneweber et al. 2006; Hattori et al. 2007) of plastid RNAs, and likely perform similar roles in the expression of normal plant mitochondrial genes. Restorer genes encoding PPR proteins might have evolved through duplication of genes encoding PPR proteins essential to normal mitochondrial gene expression, followed by divergence of mitochondrial targets (Touzet and Budar 2004; Geddy and Brown 2007).

21.3.4 Transgenic Approaches to CMS

Novel transgenic strategies have recently been applied to induce CMS. One strategy exploits genetic transformation of maternally inherited plastid genomes. Another modifies mitochondrial genome organization via nuclear genome transformation.

21.3.4.1 CMS via Plastid Transgenes

There is currently no technology for the genetic transformation of plant mitochondrial genomes (Pelletier and Budar 2007), but the genetic transformation of plastid genomes provides a route to engineering CMS. Ruiz and Daniell (2005) transformed the tobacco plastid genome with a bacterial gene (*phaA*) encoding the enzyme β-ketothiolase. In plants expressing a plastid *phaA* gene, tapetal cells undergo premature degeneration, anthers are misshapen, and pollen is collapsed. These plants are female-fertile, producing uniform pollen-sterile progenies after fertilization with wild-type pollen. β-ketothiolase might disrupt fatty acid metabolism critical to tapetal cell function and pollen development. Tapetal cell abnormalities often appear to be the basis of male sterility in mitochondrial CMS systems (reviewed in Hanson and Bentolila 2004; Chase 2007). Full restoration of male fertility has not been achieved for CMS conditioned by plastid-expressed *phaA*, but this example lays the groundwork for plastid-based CMS. While plastid transformation is not routine in all crops, advances have been made in some plants (Daniell et al. 2005; Okumura et al. 2006; C.W. Liu et al. 2007; Wurbs et al. 2007). It must be remembered, however, that plastid inheritance is paternal or bi-parental in a number of plant species (Hagemann 2004).

21.3.4.2 CMS via Nuclear Transgenes

The plant mitochondrial genome has a complex, multipartite structure that is under the control of nuclear genes (reviewed in Kubo and Newton 2008). The Arabidopsis nuclear gene *MSH1* encodes an organelle-targeted homolog of the *Escherichia coli* mismatch repair protein MutS. Mutations in *MSH1* condition mitochondrial genome rearrangements (Abdelnoor et al. 2003). The down-regulation of tobacco and tomato *MSH1* genes by an RNA interference (RNAi) transgene conditions mitochondrial genome rearrangements associated with male sterility. Male-sterile phenotypes are maternally inherited, and genetically stable following removal of the inducing RNAi construct via Mendelian segregation (Sandhu et al. 2007). This strategy potentially allows for the development of stable non-transgenic CMS systems in any plant amenable to genetic transformation. It remains to be seen whether conventional genetic screens will identify fertility restoration genes for these novel CMS systems.

21.4 Nuclear-Encoded Male Sterility Systems

21.4.1 Nuclear Male Sterility Genes

Natural nuclear mutations that impair pollen and anther development, but do not disturb female reproductive development, have been described in more than 175 plant species (Kaul 1988). Male-sterile mutants can be defined as structural (physical barriers preventing successful self-fertilization), sporogenous (pollen abortion), and functional (failure of anther dehiscence). In addition, various induced mutant approaches have been exploited to disrupt the complex network of developmental pathways involved in pollen development, anther dehiscence, and pollen germination (Chaudhury 1993; Williams 1995; Horner and Palmer 1995; McCormick 2004; X. Liu et al. 2007). Many such mutants have been evaluated for their adaptability with respect to F1-hybrid breeding, and are or have been used in breeding programs (Horner and Palmer 1995; Budar and Pelletier 2001; Perez-Prat and van Lookeren Campagne 2002; Atanassova 2007). They are referred to as genic male sterility (GMS) or nuclear male sterility (NMS) systems. Of practical importance is the environmental GMS found in rice and other crops (Ku et al. 2001; S. Li et al. 2007) that depends on stimuli like temperature or photoperiod, and has been used to develop hybrid rice varieties in the past.

Although nuclear male-sterile plants are valuable in elucidating pollen development, their application in breeding programs is limited, as no homozygous male-sterile populations can be produced (Williams 1995). In most systems, male-sterile plants must be propagated through pollination with male-fertile plants, which leads to segregation in the progeny and hampers the maintenance of the female (i.e., male-sterile) parent (Perez-Prat and van Lookeren Campagne 2002). In a few cases,

both a male sterility gene and a restorer gene are available, and three-line systems of F1-hybrid seed production have been applied to create 100% male-sterile populations (Lu et al. 2004), but the complexity of genotypes involved requires tremendous efforts. In contrast, transgenes offer virtually unlimited options to create male sterility traits, and several transgenic nuclear male sterility systems targeting pollen have been developed.

As many crop species are now amenable to nuclear transformation, genetically engineering nuclear male sterility is an important alternative to CMS and non-GMO NMS. To overcome the limitations of NMS, in particular the problem of segregation of the male sterility trait, numerous strategies have been devised to deploy and regulate nuclear genes for the purposes of creating and propagating uniformly pollen-sterile plant populations. Reversible male sterility (also called conditional male fertility) systems avoid the elimination of segregating male-fertile plants. Moreover, a novel technology adds the creation of homozygous male-sterile plants to reversible male sterility.

21.4.2 Genetically Engineered Nuclear Male Sterility

Due to the remarkable complexity of reproductive development (McCormick 2004; Scott et al. 2004), numerous approaches have been tried to induce nuclear male sterility. Male sterility phenotypes, i.e., abortion of pollen, can occur at different stages during pollen development, ranging from meiosis until dehiscence or pollen germination (Chaudhury 1993; Williams 1995; Perez-Prat and van Lookeren-Campagne 2002; Gleba et al. 2004). Both engineering natural plant genes and introducing genes from other sources, e.g., fungi and bacteria, have led to male sterility (Williams 1995; Budar and Pelletier 2001; Perez-Prat and van Lookeren-Campagne 2002; Gleba et al. 2004).

21.4.2.1 Restoration and Elimination of Segregating Male-Fertile Plants

The tapetum surrounds the developing pollen until first pollen mitosis, and plays a crucial role in the formation of microspores (Wilson and Yang 2004). Premature degeneration of the tapetal cells inevitably leads to pollen abortion. Severe defects in pollen development have been observed when toxic genes like *barnase* or *diphtheria toxin A* were transferred into plants under control of cell type-specific promoters (Mariani et al. 1990; Guerineau et al. 2003; Liu and Liu 2008). Similarly, when the tobacco-derived *TA29* promoter (Koltunow et al. 1990) was silenced in transgenic tobacco by RNAi, the tapetal cells degenerated and pollen aborted (Nawaz-ul-Rehman et al. 2007). Targeting the tapetum allowed for the development of the only commercialized transgenic male sterility, SeedLink^{TM}, used in canola and based on the work of Mariani et al. (1990). Pollen sterility resulting from the selective destruction of tapetal cells in transgenic tobacco and canola plants was

engineered by the tapetum-specific expression of a natural cytotoxic ribonuclease, the *barnase* gene of *Bacillus amyloliquefaciens* under control of the *TA29* promoter.

SeedLink^{TM} sterility was reversed when the male-sterile plants were crossed with transgenic male-fertile plants expressing the barnase inhibitor barstar, which protected the tapetal cells from degradation via barnase-barstar protein complex formation (Mariani et al. 1992). To enhance the expression of the restorer (*barstar*) gene in male-sterile *TA29-barnase* lines of *Brassica juncea*, two promoters (*TA29* from tobacco, and *A9* from *Arabidopsis thaliana*) were used to express, and thereby extend, the temporal expression of two independent wild-type and codon-modified transcriptional units of *barstar* (Bisht et al. 2004). The two different versions of the *barstar* gene presumably also minimized co-suppression effects.

The *barnase/barstar* F1-hybrid technology was faced with the general problem inherent in NMS, i.e., segregation of male-sterile and male-fertile plants in maintenance breeding of the male-sterile line. Linking the *barnase* gene to the bialaphos resistance (*bar*; Reynaerts et al. 1993) or mutated acetolactate synthase (*ALS*; Ray et al. 2007) herbicide resistance genes allowed to eliminate male-fertile plants by herbicide treatments. Still, excess seeds needed to be sown to avoid yield penalties.

Another strategy to remove unwanted fertile plants in barnase-mediated NMS was a two-component system based on independent transgenic tomato lines carrying inactive partial barnase peptides (Burgess et al. 2002). Expression of partial peptides alone did not affect pollen viability, allowing for the maintenance of inbred parents in a homozygous state by selfing. When the inbreds were crossed, barnase activity was reconstituted by complementation, and destroyed pollen in the progeny. A recent variation on this theme was a system that combined the *barnase* gene with mutated *ALS* genes (Gils et al. 2008). Male sterility and linked herbicide resistance resulted from the functional complementation of split *barnase* and *ALS* fragments (respectively) fused to trans-splicing inteins, and located on two homologous chromosomes. This system acts, by design, only in heterozygous plants that combine all elements. Plants lacking one of the elements, e.g., after hybridization with a wild-type inbred line, are pollen-fertile.

Inactivation of transcription factors has frequently led to male sterility (e.g., Preston et al. 2004), but practical application has been discussed in only in a few cases. The transcription factor AtMYB103 is essential to tapetum development in Arabidopsis. An insertion mutant disrupting the *AtMYB103* gene led to premature vacuolation and degeneration of the tapetum, and complete disruption of pollen development (S.F. Li et al. 2007). Expression of the *MYB103* gene by a strong anther-specific promoter successfully restored pollen fertility. Based on the occurrence of *MYB103* homologs in important crop plants like rice, barley, and canola, the authors suggested the use of the system for F1-hybrid seed production. Over-expression of another transcription factor, MYB26, caused ectopic lignification that included the anther wall, causing failure of the anthers to dehisce (functional male sterility; Yang et al. 2007). Pollen was viable in the over-expression lines, and could be released mechanically from the anthers. Consequently, restoration was not required for the reversal of MYB26-mediated pollen sterility (Steiner-Lange et al. 2003; Yang et al. 2007).

21.4.2.2 Reversible Male Sterility

Pollen requires for its development nutrients and signals that are provided by the tapetum and other tissues of the anther. The nutritional requirements of developing pollen became evident when isolated microspores were able to develop into mature, fertile pollen under appropriate culture conditions (Benito Moreno et al. 1988; Tupý et al. 1991), and when pollen tube growth was enhanced upon addition of flavonols to in vitro matured pollen (Ylstra et al. 1992). Down-regulation or over-expression of enzymes involved in the synthesis of metabolites that are essential for pollen development offer numerous possibilities to engineer male sterility. Application of the missing nutrient should lead to restoration of pollen fertility (reversible male sterility).

Metabolic engineering of primary metabolites has targeted glutamine, carbohydrates, and pyruvate. The amino acid glutamine is essential for pollen development (Benito Moreno et al. 1988). Transferring into tobacco plants a gene driven by the tapetum-specific promoter *TA29* and encoding a dominant-negative version of glutamine synthetase (dnGS) resulted in male sterility (Ribarits et al. 2007). Spraying plants with glutamine restored male fertility. Anthers of the male-sterile lines contained viable microspores, and restoration was also achieved by isolating the microspores, maturing these in vitro in the presence of glutamine, and using the in vitro matured pollen for pollination of emasculated flowers in situ (Touraev and Heberle-Bors 1999). A similar strategy has been tried in tobacco plants. Here, knocking down an extracellular invertase gene by an anti-sense construct resulted in carbohydrate deficiency of pollen (Goetz et al. 2001), but no seeds were obtained after pollination with pollen matured in vitro in a sugar-containing medium. Down-regulation of pyruvate dehydrogenase was used to mimic CMS, and led to hypertrophy and vacuolation of the tapetal cells (Yui et al. 2003). However, no restoration strategies were devised in this case.

Male sterility resulting from disruption of flavonoid biosynthesis by anti-sense suppression, co-suppression, or mutations (Taylor and Jorgensen 1992; van der Meer et al. 1992; Napoli et al. 1999) was restored when flavonols were applied manually during pollination (Mo et al. 1992; Ylstra et al. 1994), or by expressing the key enzyme in flavonoid biosynthesis, chalcone synthase (Napoli et al. 1999). Exploiting the metabolic similarities of stilbene synthase and chalcone synthetase, tapetum-specific expression of grapevine stilbene synthase was shown to cause male sterility in tobacco, which could partially be reversed by application of 4-coumarate, resveratrol, and flavonols (Fischer et al. 1997). Jasmonic acid biosynthesis is also essential for pollen development and anther dehiscence, and suppression of several enzymes involved in this pathway has been shown to lead to male sterility that can be reversed by the application of linolenic or jasmonic acid (McConn and Browse 1996; Stintzi and Browse 2000; Ishiguro et al. 2001; Park et al. 2002).

Impaired reception and signal transduction of hormones in pollen, and reversal by exogenous application of growth regulators such as kinetin, gibberellic, or jasmonic acid have also been employed to engineer male sterility (Huang et al. 2003; Al-Ahmad and Gressel 2005). No restoration, however, has been reported for tobacco lines carrying an over-expressed ethylene receptor (Ishimaru et al. 2006).

21.4.2.3 Induced Male Sterility

An alternative to reversible male sterility is induced male sterility. The strategy described by Kriete et al. (1996) involved transgenic tobacco plants expressing the bacterial *argE* gene specifically in the tapetum. When these plants were treated with the non-toxic compound *N*-acetyl-phosphinothricin, it was converted into the herbicide phosphinotricin, thereby destroying the tapetal cells. F1-hybrid breeding based on induced male sterility employs two male-fertile inbred lines, one of which is sprayed with *N*-acetyl-phosphinothricin for F1-hybrid seed production.

21.4.2.4 Homozygous Nuclear Male-Sterile Lines

An F1-hybrid seed production system that combines reversible male sterility with a general strategy to produce homozygous male-sterile plants is based on the metabolic engineering of glutamine, as described above (Ribarits et al. 2007). To overcome segregation of the nuclear *TA29-dnGS* gene in maintenance breeding of the male-sterile inbred line, viable microspores contained in its anthers were recovered and used to produce doubled haploid plants through in vitro microspore culture in the presence of glutamine (Touraev and Heberle-Bors 1999). Being 100% homozygous, these doubled haploid plants did not segregate into male-sterile and male-fertile plants in the offspring, but produced only male-sterile plants. Doubled haploids are widely used in plant breeding, for example, in barley, wheat, and canola, not only in selection and combination breeding, but also as inbred lines in F1-hybrid breeding (Forster et al. 2007).

F1 hybrids produced by this method were male-sterile, and could be used for crops in which the vegetative parts of the plants are used. To make this technology applicable also for F1-hybrid breeding in seed crops, the microspore-specific *NTM19* promoter was employed to drive the *dnGS* gene. Transgenic plants containing one *NTM19-dnGS* locus were male-fertile, but 50% of the pollen (the pollen carrying *NTM19-dnGS*) was not viable. To achieve complete male sterility, viable microspores produced by these lines were isolated and cultured in vitro, in the presence of glutamine, to produce homozygous doubled haploid plants. Doubled haploid lines homozygous for the *NTM19-dnGS* gene were indeed male-sterile, and did not segregate into male-sterile and male-fertile plants in the offspring. To maintain the male-sterile line, plants were sprayed with glutamine, an innocuous chemical that restored pollen fertility. Crossing these lines with male-fertile wild-type lines produced heterozygous F1 lines that were male-fertile, due to the presence of 50% wild-type pollen.

In conclusion, this technology combined essential features of F1-hybrid breeding, i.e., a method to create inbred lines (doubled haploidy), a method to produce F1-hybrid seeds (male sterility), and a method to maintain nuclear male-sterile lines as homozygotes (fertility restoration by reversible sterility and doubled haploids).

21.5 Summary and Future Prospects

The past 15 years have seen a virtual explosion in attempts to engineer male sterility for the breeding of F1 hybrids. Breeders are today faced with a variety of options. Not all, however, apply to any given crop species, and some of the new published methods lack a complete strategy for F1-hybrid seed production. Given the choice between CMS and NMS, CMS provides the benefit that male-sterile lines can easily be propagated due to the maternal inheritance of mitochondrial and plastid genomes. CMS is, however, difficult and time-consuming to introduce in a new species, and even more so the corresponding restorer genes. NMS can more easily be introduced, particularly since today many crop species are amenable to nuclear transformation. In addition, the large number of genes expressed in pollen, and the availability of different molecular and genetic tools to engineer gene expression offer numerous possibilities to block pollen development, often in a conditional manner. However, NMS faces the problem of segregation during maintenance breeding. Yet, also for this problem recent developments have offered solutions. Given that most breeders today are familiar with GMOs and doubled haploids, F1-hybrid seed technologies that combine methods to create inbred lines by doubled haploidy, to produce F1-hybrid seeds by NMS, and to maintain nuclear male-sterile lines as homozygotes by fertility restoration through reversible sterility and doubled haploids seem to be a promising path to the future. Another is induced male sterility.

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Chapter 22
Self-Incompatibility Systems in Flowering Plants

P. Kaothien-Nakayama, A. Isogai, and S. Takayama

22.1 Introduction

Sexual reproduction is a primary reproductive system used by flowering plants to increase genetic diversity of their offspring. However, the majority of flowering plants are prone to self-pollination because they are hermaphrodite, producing both male and female reproductive organs on the same flower. Self-incompatibility (SI) systems are genetic systems evolved by plants with hermaphroditic flowers to differentiate and reject self-pollen, thereby preventing self-fertilization, and promoting outcrossing. These SI mechanisms work by controlling the interaction between the pistil (female organ) and the pollen (male gamete) at organellar, cellular, and molecular levels.

SI systems have evolved several times during the course of the evolution of flowering plants, leading to a diverse array of SI mechanisms identified to date (Charlesworth et al. 2005; Shimizu et al. 2008). SI systems are categorized into heteromorphic SI and homomorphic SI systems. In a heteromorphic SI system, flowers from different incompatibility groups have different morphology. This SI system is also called a heterostylous SI system, because differences in style length and anther positioning define the outcome of SI interaction between the pollen and the pistil. There are two types of heterostylous SI, namely, distylous SI having two flower forms (short style with high anthers, and long style with low anthers), and tristylous SI having three flower forms (short style with middle and high anthers, middle style with low and high anthers, and long style with low and middle anthers; de Nettancourt 2001). Style length and anther positioning are controlled genetically by a dominance relationship between alleles in a single *S*-locus in distylous SI plants (Lewis 1949), and by dominance relationship between alleles in two loci,

P. Kaothien-Nakayama, A. Isogai, and S. Takayama
Graduate School of Biological Sciences, Nara Institute of Science and Technology, Nara 630-0192, Japan
e-mail: takayama@bs.naist.jp

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S and *M*, in tristylous SI plants (Mather 1943). In both distylous and tristylous SI, the rule of SI interaction is simple. A compatible interaction occurs only when pollen is derived from the anther having the same height as the recipient stigma.

In the homomorphic SI system, flowers from different incompatibility groups have identical floral form, and self-rejection is controlled by a multi-allelic *S*-locus (de Nettancourt 2001). Male and female *S*-determinants of SI, with expressions displaying dominance or co-dominance relationships, are encoded within this locus, and pollen rejection occurs when the same *S*-allele (more usually called *S*-haplotype) is expressed in both the pollen and the pistil. The homomorphic SI system is classified further, based on a genetic system that controls the SI phenotype of pollen, into the gametophytic SI (GSI) and sporophytic SI (SSI) systems (Fig. 22.1, Table 22.1). In the GSI system, the SI phenotype of the pollen is

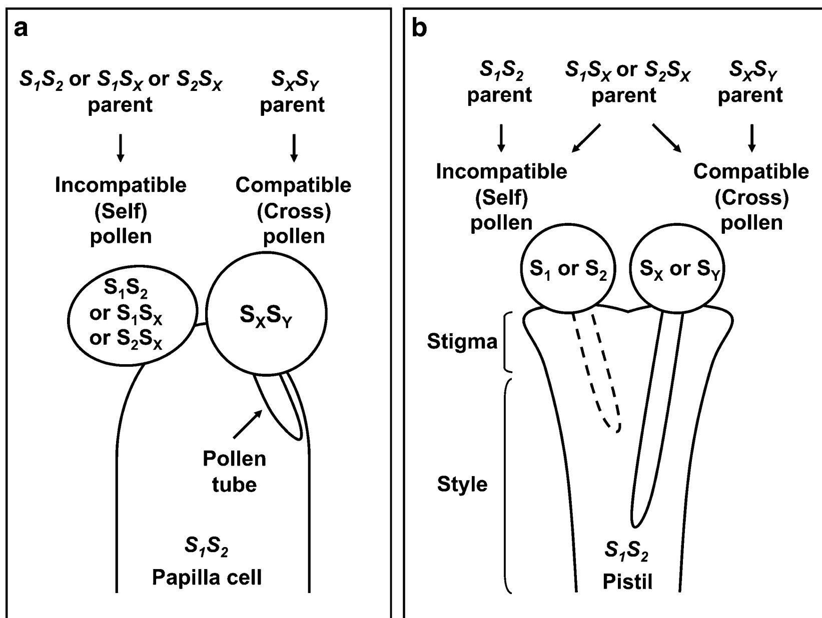

Fig. 22.1 Two types of genetically controlled system in homomorphic self-incompatibility. **a** Sporophytic self-incompatibility (SI) system in Brassicaceae. In this system, SI phenotype of the pollen is determined by the diploid genome of its parent. Pollen is incompatible (recognized by the recipient pistil as self-pollen) when its parent expresses both (S_1S_2) or one (S_1S_x or S_2S_x) of the *S*-haplotypes expressed by the diploid recipient pistil (S_1S_2). Pollen is compatible (accepted by recipient pistil as cross-pollen) only when its parent expresses a pair of *S*-haplotype (S_XS_Y) completely different from that of the recipient pistil (S_1S_2). The self-pollen is usually rejected at the step of pollen hydration on the surface of the stigmatic papilla cell. **b** Gametophytic self-incompatibility (GSI) system in Solanaceae, Rosaceae, and Scrophulariaceae. In this system, SI phenotype of the pollen is determined by its own haploid genome. Pollen is compatible when its own *S*-haplotype (S_x or S_y) is different from those expressed by the diploid recipient pistil (S_1S_2). Both incompatible (self)- and compatible (cross)-pollen hydrate and germinate tubes, but only the cross pollen tube continues to grow through the entire distance of the style, and reaches the ovule. The growth of the self pollen tube comes to a stop within the style as a result of the SI response

Table 22.1 Comparison of self-incompatibility (SI) systems that operate in different families of economically important crops

	Brassicaceae	Solanaceae, Rosaceae, Scrophulariaceae
SI system	SSI	S-RNase based GSI
Type of stigma and mature pollen	Dry stigma, trinucleate mature pollen	Wet stigma, dinucleate mature pollen
Site of self pollen rejection	Papilla cells of the stigma	Style
Rejection mechanism	Blockage of water or supportive factors from papilla cell to pollen	Pollen tube RNA degradation
Female determinant	SRK (receptor kinase)	S-RNase (ribonuclease)
Male determinant	SP11/SCR (small cysteine-rich protein)	SLF/SFB (F-box protein)
SI activation mechanism	*S*-haplotype-specific interaction between SRK and SP11/SCR	Not clear

determined by its own haploid genotype, while in the SSI system, the SI phenotype of the pollen is determined by the genotype of its diploid parent (de Nettancourt 2001).

SI is a very important agricultural trait for breeding hybrid varieties of economically important plant families, including the Brassicaceae (broccoli, Brussels sprouts, cabbage, cauliflower, mustard, rapeseeds, and turnip), Rosaceae (apple, apricot, cherry, pear, and rose), Scrophulariaceae (snapdragon), and Solanaceae (petunia, potato, tobacco, and tomato). In this chapter, the mechanisms of homomorphic SI systems that operate in these plant families, and the potential usage of these SI systems in plant breeding programs are described. For brevity, the terms self- and cross-pollen are sometimes used in this chapter as substitute for incompatible and compatible pollen, respectively. Therefore, the term self-pollen used here always means pollen with SI phenotype that is incompatible with the recipient stigma, and does not simply mean pollen from the same plant. Likewise, the term cross-pollen used here always means pollen with SI phenotype that is compatible with the recipient stigma, and does not simply mean pollen from a different plant.

22.2 Sporophytic Self-Incompatibility in Brassicaceae

22.2.1 Physiological Aspect

Mature pollen grains that land on the surface of the stigma are usually dehydrated, and need to be hydrated to activate their metabolism. Normally, in Brassicaceae, compatible pollen grains adhere strongly to the secretory epidermal cells of the stigma (papilla cells) within 15 min after contact, and become fully hydrated within 30 to 90 min (Stead et al. 1980; Dickinson 1995). With dry-type stigma, Brassicaceae plants usually reject self-pollen by interfering with this hydration process. Under some environmental conditions, or for some *S*-haplotype combinations,

hydration of self-pollen and subsequent pollen tube germination occur. However, the penetration of the self pollen tube into the wall of the papilla cell is later inhibited (Dickinson 1995; Dearnaley et al. 1999).

Pollen-pistil interaction in the Brassicaceae SI system was found to be localized and reversible. An individual papilla cell responded correctly to self and cross pollen grains placed alongside each other on its surface (Sarker et al. 1988), and pollen grains positioned between self and cross papilla cells adhered only to the cross papilla cell and became hydrated from it (Hiscock et al. 1994). In support of these observations, localized secretion and polarized arrangement of the actin cytoskeleton inside the papilla cell were observed toward the point of cross pollen contact, while disorganization, and most probably depolymerization, of the actin cytoskeleton associated with the apical vacuole network was observed underneath the site of self pollen contact (Elleman and Dickinson 1994; Iwano et al. 2007). Pollen placed on an incompatible stigma for 10 to 20 min can be resurrected when transferred to its compatible stigma, showing that rejection within 1 to 20 min does not result in deterioration of the rejected pollen (Kroh 1966). These observations suggest that blockage of localized transport of supplies is the mechanism used by the papilla cell to reject self-pollen. Pollen grains were also hydrated faster when placed on an immature stigma, or on a stigma that was pretreated with an inhibitor of protein synthesis or protein glycosylation, compared to when placed on an untreated mature stigma (Sarker et al. 1988). These observations imply that pollen-papilla interaction in Brassicaceae SSI might involve activity of glycosylated protein(s) present in mature papilla cells.

22.2.2 *Cloning of S*-Genes, and the Nature of *S*-Gene Products

The *S*-locus is defined as a hyper-variable region that contains the male and the female determinants of SI. The *S*-locus spans a region of about 50 kb in most *S*-haplotypes of *Brassica rapa* (turnip rape), and more than 200 kb in *B. oleracea* (kale; Boyes and Nasrallah 1993). As a result of two decades of molecular analysis of the *S*-locus, the genes that encode the male and the female determinants of SI were identified.

22.2.2.1 Female Determinant of SI

The first important clue that lead to the identification of the female SI determinant came from the identification of an *S*-haplotype-specific antigen from the stigma that co-segregated with the *S*-locus, based on isoelectric focusing and immunochemical detection (Nasrallah and Wallace 1967; Nishio and Hinata 1977; Nou et al. 1993). Cloning of the gene encoding this antigen, and biochemical analysis of the gene product have shown that this antigen is a secreted glycoprotein, named *S*-locus glycoprotein (SLG), which accumulates in the wall of papilla cells to a high concentration (Nasrallah et al. 1985; Takayama et al. 1987; Kandasamy et al.

1989). The similarity between the sequence of SLG and the sequence of the extracellular domain of *ZmPK1*, a receptor-like kinase in maize (Walker and Zhang 1990), led to the isolation of the other *S*-gene product, the *S*-locus receptor kinase (SRK; Stein et al. 1991). SRK structure consists of an SLG-like extracellular domain, a transmembrane domain, and a cytoplasmic serine/threonine kinase domain. Recombinant SRK proteins were found in the microsome fraction, and were capable of autophosphorylation in trans on serine and threonine residues in the absence of stigma extract (Giranton et al. 2000). SRK was also localized to the plasma membrane when transiently expressed in the protoplasts of tobacco cultured cells (Kakita et al. 2007). Comparison of *SLG* and *SRK* sequences from different haplotypes has confirmed that these genes exhibit allelic sequence diversity (Stein et al. 1996). Sequence analysis also revealed substantial similarity between the sequence of the extracellular domain of SRK, and the sequence of SLG of the same haplotype, while significant differences were found between *SRK* and *SLG* from a different *S*-haplotype (Goring and Rothstein 1992; Delorme et al. 1995). Detailed analyses of *SLG* and *SRK* expression have revealed that both genes are expressed in papilla cells, and both transcripts were accumulated to the maximum level immediately before the occurrence of pollen anthesis (Stein et al. 1996).

Although the data described above suggest that both SLG and SRK could be the female *S*-determinant, analyses of the *S*-locus revealed that SLG was not present in some *B. rapa* and in *Arabidopsis lyrata*, all of which possess an intact self-incompatible phenotype (Kusaba et al. 2001; Suzuki et al. 2003), while SRK protein was not present in some self-compatible *Arabidopsis thaliana* (Stein et al. 1996; Sherman-Broyles et al. 2007). Moreover, in a gain-of-function experiment, transgenic *B. rapa* S_{60} plants expressing SRK_{28} (*SRK* of S_{28} haplotype) acquired the S_{28} specificity in their stigmas (rejecting S_{28} pollen), while those expressing SLG_{28} did not (Takasaki et al. 2000). These data together suggest that SRK alone functions as a female determinant of SI in the Brassicaceae.

22.2.2.2 Male Determinant of SI

After the finding that the female determinant of SI is a receptor-like kinase, the focus of research was to identify its pollen-borne ligand, which most probably also functions as the male determinant of SI. Because of the sporophytic nature of the pollen SI phenotype, researchers searched for molecules with male-determinant activity in the pollen coat, deposited onto the surface of pollen grains by the sporophytic tapetal cells of the anther. The proof that the male-determinant activity actually resides in the pollen coat came with the result of a pollen bioassay wherein cross-pollen was rejected on the stigma pretreated with the coat of self-pollen, and self-pollen was accepted on the stigma pretreated with the coat of cross-pollen (Stephenson et al. 1997). Subsequent fractionation analyses of the pollen coat have revealed that the male-determinant activity exists in its water-soluble small-protein fraction (<10 kDa; Stephenson et al. 1997). Finally, the gene that encodes this pollen coat protein was identified by sequence analysis of the *S*-locus region, and by

a polymorphic gene search using fluorescent differential display (Schopfer et al. 1999; Suzuki et al. 1999; Takayama et al. 2000). The protein was named *S*-locus protein 11 (SP11), or *S*-locus cysteine-rich protein (SCR).

SP11/SCR is a small, cysteine-rich protein secreted by the tapetum cells of the anther, and deposited on the pollen coat during pollen maturation (Schopfer et al. 1999; Takayama et al. 2000; Iwano et al. 2003). This sporophytic nature of SP11/SCR expression explains the sporophytic nature of the SI phenotype of Brassicaceae pollen. Sequence analyses have revealed that SP11/SCR alleles are highly polymorphic (20–94% amino acid identity), even more than those amongst SRK alleles (65–98% amino acid identity). The few conserved features in mature SP11/SCR protein are the eight conserved cysteine residues (though some alleles lack the C-terminal cysteine), a glycine residue between the first and the second cysteine, and an aromatic residue between the third and the forth cysteine (Watanabe et al. 2000). Solution structure analysis revealed that SP11/SCR folds into an α/β sandwich structure similar to those of plant defensins, and that the conserved glycine and aromatic residues are important for SP11/SCR structural integrity (Mishima et al. 2003). The function of SP11/SCR as the sole male determinant of Brassica SSI was proved by a loss-of-function study, where loss of SP11/SCR expression in a homozygous plant conferred loss of SI, and by gain-of-function studies, in which transgenic pollen acquired the *S*-specificity of the introduced SP11/SCR, and cross pollen grains were rejected on stigmas pretreated with recombinant or synthetic self SP11/SCR (Schopfer et al. 1999; Shiba et al. 2001).

22.2.2.3 Interaction Between Male and Female SI Determinants

The above findings that SRK, a plasma membrane receptor-like kinase, and SP11/SCR, a small secreted, cysteine-rich protein, are the female and the male determinants of SI suggest that *S*-haplotype-specific receptor-ligand interaction between the two might regulate the recognition step of Brassicaceae SSI. In fact, *S*-haplotype-specific interaction between SRK and SP11/SCR was demonstrated in an in vitro pull-down experiment using recombinant fusion proteins (Kachroo et al. 2001), and in a semi in vivo immunoprecipitation experiment where antibody raised against SRK pulled down a cross-linked stigma protein complex that contained SRK, radio-labeled SP11/SCR, and a 60-kDa protein detectable by anti-SRK antibody (Takayama et al. 2001). This 60-kDa protein was later identified as a truncated form of SRK (tSRK), which contained the extracellular domain, the transmembrane domain, and a part of the juxtamembrane domain (Shimosato et al. 2007). In the same study, a transiently expressed tSRK was found to exhibit high-affinity binding to SP11/SCR, while the SLG and the alternatively spliced SRK, containing only the extracellular domain (eSRK), exhibited no high-affinity binding to SP11/SCR. This is in agreement with the finding that SRK formed an oligomer in the absence of ligand in microsomes of insect cells and in plants, while SLG and eSRK were mostly monomeric (Giranton et al. 2000). Interestingly, the artificial dimerized form of the soluble eSRK protein (eSRK-HLH-ZIP fusion

protein) could interact with SP11/SCR in vitro (Shimosato et al. 2007). These findings suggest that SRK dimerization, supported by a membranous environment, is crucial for the formation of the SP11/SCR-SRK complex. Attempts were also made to map the protein domains responsible for SP11/SCR-SRK interaction, and the domains responsible for SRK oligomerization. Solution structure analysis suggested that the hyper-variable region of SP11/SCR might form a loop that extends out from the body of the protein, and serves as the SRK binding site (Mishima et al. 2003). Using a surface-display system in yeast, ligand-independent SRK dimerization was found to depend on the two regions within the extracellular domain of SRK, these being the C-terminal PAN APPLE domain, and the EGF-like domain (Naithani et al. 2007). In support of its purposed functional role in SI, SRK was found to be autophosporylated rapidly after interacting with SP11/SCR of the same haplotype (Takayama et al. 2001).

22.2.2.4 Dominance and Co-Dominance Relationships Between *S*-Gene Products

Dominance and co-dominance relationship between *S*-alleles exist in Brassicaceae SSI. These relationships are nonlinear, and can be different in pollen and pistil (de Nettancourt 2001). Because *S*-specific phenotypes of both the pollen and the pistil are controlled by the diploid genome of the parental plant, dominance and co-dominance relationships amongst haplotypes significantly influence the final outcome of SI interaction between the two. In the well-characterized *S*-haplotypes of *Brassica*, the co-dominance relationship is more common than the dominance relationship, and when the dominance relationship occurs, it is usually confined to the pollen (Nasrallah 2002; Watanabe et al. 2003). This makes sense, because the dominance relationship amongst the male determinants would confer a greater chance for mating to the pollen, while the co-dominance expression of the female *S*-determinant would confer a stronger selection against self-pollen to the pistil. Interestingly, while dominance relationships between SRK alleles are controlled at a post-translational level (Hatakeyama et al. 2001), those between SP11/SCR alleles are controlled at the transcription level (Shiba et al. 2002).

Extensive analyses of the dominance relationships between *S*-haplotypes in *B. rapa* (turnip rape) have revealed a certain dominance hierarchy in determining the SI phenotype of pollen, where the allelic partner in *S*-heterozygotes affects the dominance relationships. The *S*-haplotypes belonging to class I (e.g., S_{52}) are dominant over those belonging to class II (e.g., S_{44}, S_{60}, S_{40}, and S_{29}), and the linear dominance relationship exists amongst class-II members (e.g., $S_{52}>S_{44}>S_{60}>S_{40}>S_{29}$). Of these *S*-haplotypes, S_{52} is always dominant over other *S*-haplotypes, S_{29} is always recessive to the others, while S_{44}, S_{60}, and S_{40} are dominant in some cases, but recessive in others (Nasrallah and Nasrallah 1993; Hatakeyama et al. 1998). This order of dominance hierarchy were further confirmed by data from expression analyses, showing that the transcript from a recessive *S*-haplotype, but not from a dominant *S*-haplotype, is always diminished in the anther

tapetum of all examined *S*-heterozygotes (Shiba et al. 2002; Kakizaki et al. 2003). Interestingly, the promoter region of a recessive *SP11/SCR* allele was specifically methylated in the anther tapetum prior to the initiation of *SP11/SCR* transcription in the *S*-heterozygote (Shiba et al. 2006). This is the first finding to suggest the involvement of epigenetics in classical Mendelism (dominance relationships), and the occurrence of tissue- and developmental stage-specific de novo methylation in plants.

22.2.3 Other Components of the SSI Pathway

As the function of SRK and SP11/SCR in SSI became evident, the focus on Brassicaceae SSI research was set on finding other components of the pathway. As a result, several components working upstream and downstream of the SP11/SCR-SRK interaction were identified, as listed below.

Two thioredoxin-h proteins, THL1 and THL2, were identified as SRK-binding partners in a yeast two-hybrid screen with a kinase domain of SRK as bait (Bower et al. 1996). The interactions between SRK and THL1/2 were found to be phosphorylation-independent, and required a conserved cysteine residue at the transmembrane domain of SRK (Mazzurco et al. 2001). Autophosphorylation of SRK was inhibited by THL1, and this inhibition could be released by the addition of pollen coat proteins derived from pollen with the same *S*-haplotype as that of the tested SRK (Cabrillac et al. 2001). In the same study, only a small portion of the SRKs was found in the phosphorylated stage in vivo, while most SRKs were found in an autophosphorylated stage in a partially purified stigma-microsome fraction, which most probably was deprived of THL1/2 proteins. Moreover, antisense expression of *THL1/2* in stigmatic tissues conferred a weak SI phenotype to the stigma of the normally self-compatible *B. napus* (rapeseeds; Haffani et al. 2004). These results suggest that THL1/2 may interact with SRK upstream of SP11/SCR-SRK interaction, and function to keep SRK in the inactive stage. The second SRK-interactor identified by yeast two-hybrid screen with SRK kinase domain is the Armadillo repeat containing protein (ARC1; Gu et al. 1998). ARC1 structure comprises the N-terminal domain (UND), a U-box domain, and an ARM-repeat domain. The function of the UND domain is unknown, the U-box mediates the interaction with E2 ubiquitin-conjugating enzyme, and the ARM repeat mediates the interaction with SRK (Samuel et al. 2006). The composition of structural domains of ARC1 implies that it might function as an E3 ubiquitin ligase, the enzyme that catalyzes the last step of the ubiquitination process. In tobacco suspension cultured cells, ARC1 actually functioned as a ubiquitin ligase that translocated to the proteasome, the site of ubiquitinated protein degradation, only in the presence of an active SRK (Stone et al. 2003). Moreover, inhibition of proteasomal proteolytic activity disrupted the SI response. In contrast to THL1/2, ARC1 interaction to SRK was found to be phosphorylation-dependent, and ARC1 was phosphorylated by SRK (Gu et al. 1998; Mazzurco et al. 2001). ARC1 is also specifically expressed in the stigma at the same time as SRK (Gu et al. 1998), and

antisense suppression of ARC1 resulted in partial breakdown of SI response (Stone et al. 1999). These results imply that ARC1 functions downstream of SP11/SCR-SRK interaction, possibly by promoting ubiquitination and proteasome degradation of papilla protein(s) during the SI response (Stone et al. 2003). Although not yet identified, the papilla protein target(s) of ARC1 is speculated to be the protein(s) that functions to support growth of the compatible pollen tube.

Different from the three components described above, the next component of *Brassica* SSI was identified by a series of genetic mapping and positional cloning of the recessive mutant gene, initially named the *modifier* (*m*) gene, responsible for the loss of stigma SI function in *B. rapa* var. Yellow Sarson (Murase et al. 2004). Position cloning of the *M* gene revealed that it encodes a member of the receptor-like cytoplasmic kinase (RLCK) family. Therefore, the gene was renamed *M-locus protein kinase* (*MLPK*; Murase et al. 2004). Sequence analysis has revealed that a missense mutation in a conserved kinase domain is responsible for the disruption of the SI response found in *B. rapa* var. Yellow Sarson. In vitro phosphorylation assays further revealed that this mutation resulted in the loss of MLPK kinase activity, as well as of its protein stability (Murase et al. 2004), and that the kinase domain of SRK could phosphorylate MLPK (Kakita et al. 2007). MLPK was found to exist in two isoforms, MLPKf1 and MLPKf2, derived from alternative first exons (AFEs) transcripts of the *MLPK* (Kakita et al. 2007). Both MLPK isoforms are expressed in stigmatic papilla cells, and are plasma membrane-localized, albeit by different mechanisms. Furthermore, both MLPK isoforms could interact with SRK at the plasma membrane of the protoplasts of tobacco suspension cultured cells. The function of MLPK isoforms in SI was confirmed when transiently expressed MLPKf1 or MLPKf2 restored the SI phenotype to the papilla cells of *B. rapa* var. Yellow Sarson, while transient expression of their mutant forms lacking motifs important for plasma membrane localization failed to do so (Murase et al. 2004; Kakita et al. 2007).

22.2.4 Working Model and Prospects

The working model for Brassicaceae SSI is shown in Fig. 22.2. This model incorporates protein components with identified functions in the SSI signal-transduction pathway, the functional relationship between these components, and the possible biochemical and physiological responses conferred by them. Based on the above-mentioned functional relationship between SRK and ARC1, and based on the observation that pollen-pistil interaction in this SI system is localized and reversible, it is possible that the downstream events of SI would involve the degradation of papilla protein(s) necessary for the compatible pathway, which work in the papilla cell to support pollen hydration/tube growth. Therefore, more information on the mechanisms of compatible pathway is required for complete understanding of the SI signaling pathway. Plants in the genus *Arabidopsis*, the well-known member of the Brassicaceae, are currently adopted as a model plant for SSI research, because of the availability of genomic information, the plant's short life cycle, and the easy

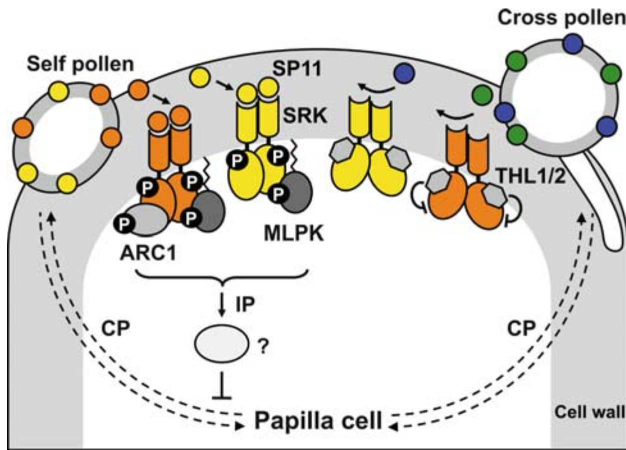

Fig. 22.2 Hypothetical model for molecular events leading to the sporophytic self-incompatibility (SSI) response in the Brassicaceae. *S*-haplotype-specific interaction between the *S*-locus receptor kinase (SRK) on the membrane of stigma papilla cell and its small, cysteine-rich ligand from the pollen coat, SP11/SCR, triggers the SI response that includes trans-autophosphorylation of the SRKs, and phosphorylation of the two SRK cytoplasmic interactors, the membrane-anchored protein kinase MLPK, and the E3 ubiquitin ligase ARC1. MLPK function is crucial for the SI response, although the event downstream from MLPK is still unknown. ARC1 might target the protein component of compatible pathway (CP) for degradation in the proteasome. In the absence of SP11/SCR, the thioredoxin-h proteins, THL1 and THL2, maintain the SRK in an inactive state to ensure the accuracy and polarity of the SI response. Different colors represent different *S*-haplotypes of the SP11s/SCRs and the SRKs. IP, incompatible pathway

transformation and handling methods. In fact, evidence supporting the current model of SSI shown in Fig. 22.2 also came from researches using *Arabidopsis*; for example, analyses of *Arabidopsis S*-locus revealed that mutations in both *SP11*/*SCR* and *SRK* rendered the genes nonfunctional in the self-compatible *A. thaliana*, and that *S*-locus of the self-incompatible *A. lyrata* contains no *SLG* (Kusaba et al. 2001). In transgenic studies, researchers have also succeeded in converting the self-compatible *A. thaliana* plant into a self-incompatible plant by introducing the functional copy of *SP11*/SCR and *SRK* genes from the self-incompatible *A. lyrata* (Nasrallah et al. 2004). Therefore, it is promising that analysis of *Arabidopsis* mutants defective in the compatible or the self-incompatible pathways will provide researchers with the information needed to understand the mechanism of Brassicaceae SSI.

22.3 S-RNase Based Gametophytic Self-Incompatibility

22.3.1 Physiological Aspect

S-RNase based GSI is the SI system used by plants in the families Rosaceae, Scrophulariaceae, and Solanaceae. In this system, haploid pollen that carries the

same *S*-haplotype as either one of those carried by the diploid pistil is rejected, and rejection occurs during pollen tube growth within the style of the pistil. Interestingly, this rejection timing coincides with the transition of pollen tube growth from the slow-growth phase to the accelerated-growth phase (Herrero and Hormaza 1996). Physiological studies have revealed that rRNAs were degraded 12 to 48 h after incompatible pollination (McClure et al. 1990), and that the tips of incompatible pollen tubes swelled before their growth ceased (de Nettancourt 1997).

22.3.2 Cloning of S-Genes, and the Nature of *S*-Gene Products

In search for the male and the female determinants of GSI, researchers have looked for pollen and pistil *S*-genes and *S*-gene products that display polymorphisms between different *S*-haplotypes. Similar to the Brassicaceae SSI, the female determinant was identified first because of its abundance in pistils.

22.3.2.1 Female Determinant of SI

The putative female determinant of GSI was first identified from the stigma and style of *Nicotiana alata* as the protein bands that co-segregated with *S*-specific pollen rejection (Bredemeijer and Blaas 1981). Consequently, the N-terminal sequence of one of the proteins was identified, and the corresponding cDNA was cloned, followed by the cloning of similar sequences from other plant families with the GSI system (Anderson et al. 1986). Sequence and functional analyses have revealed that this style protein is a 30-kDa glycoprotein with sequence similarity to a ribonuclease. Therefore, the protein was named S-RNase (McClure et al. 1989). This finding, together with the findings that rRNA were degraded 12 to 48 h after incompatible pollination, and that S-RNase activity was required for self pollen rejection (McClure et al. 1990), strongly implicated the role of ribonuclease activity in the mechanism of GSI. Analyses of *S-RNase* sequences from different haplotypes and genetic studies later revealed that *S-RNases* were highly polymorphic and co-segregated with the *S*-locus. Results from in situ hybridization and expression analysis showed that S-RNases are expressed specifically in stylar tissues, secreted into the extracellular matrix (ECM) around the tissues, and accumulated there to a high concentration (10 to 50 mg S-RNase/ml of ECM; Cornish et al. 1987; Anderson et al. 1989).

The definitive proof that S-RNase is the female determinant for this GSI system came from several functional analysis experiments in transgenic plants, and from analyses of S-RNases in naturally occurring self-compatible mutants. Site-directed mutagenesis experiments showed that the ribonuclease activity of the *Petunia* S-RNase is required for its SI function (Huang et al. 1994). Results from pollination tests in transgenic *Petunia* plants expressing antisense *S-RNase*, and from *Petunia* and *Nicotiana* plants expressing *S-RNase* of different haplotypes, confirmed that

S-RNase activity is required for pistil SI function, and that S-RNase alone could control specificity of SI in the pistil (Lee et al. 1994; Murfett et al. 1994). The normally self-incompatible *Malus domestica* (apple) transformed with an extra copy of its own *S*-gene became self-compatible as a result of co-suppression that resulted in an absence of S-RNase protein in the pistil (Broothaerts et al. 2004). A naturally self-compatible accession of *Lycopersicon peruvianum* (wild tomato) was found to produce a catalytically inactive S-RNase (Royo et al. 1994). The self-compatible phenotypes of *Pyrus pyrifolia* (Japanese pear) and *Prunus dulcis* (almond) result from the loss of S-RNase activity (Sassa et al. 1997).

Analyses of the primary structures of solanaceous (Ioerger et al. 1991) and rosaceous S-RNases (Ishimizu et al. 1998) revealed that they share some conserved and variable regions; solanaceous S-RNases have five conserved regions (C1–C5) and two hyper-variable regions (HVa and HVb), while rosaceous S-RNases have similar structures except for the lack of the C4 and the HVb regions. The conserved regions most likely function as a catalytic domain, while the HV regions are thought to have allelic-specific function. Furthermore, X-ray crystallographic analyses of solanaceous (Ida et al. 2001) and rosaceous S-RNases (Matsuura et al. 2001) showed that both possess similar three-dimensional structures of eight helices and seven β-strands with a folding topology typical for the RNase T2 family. The three-dimensional structures also suggested that the HV regions are exposed on the surface of the S-RNase molecules, making these accessible to the solvent, and therefore could possibly interact with the male *S*-determinant. The function of the HV regions as that responsible for the specificity of S-RNase activity was confirmed by a domain-swapping experiment, where modification of four amino acid residues in the S_{11}-RNase (S-RNase of S_{11} haplotype) to match those in the S_{13}-RNase was sufficient to completely replace the S_{11} phenotype with the S_{13} phenotype (Matton et al. 1997). Interestingly, when only three amino acid residues of the S_{11}-RNase were replaced by those of the S_{13}-RNase, the resulting chimeric S-RNase displayed a dual-specific phenotype, i.e., pistil expressing the chimeric $S_{11/13}$-RNase could reject both S_{11} and S_{13} pollen (Matton et al. 1999). These results demonstrated that only a few amino acid changes are needed to create new haplotypes. Imaging evidence suggested that S-RNases enter pollen tubes in a non-*S*-specific manner (Luu et al. 2000), that S-RNases are compartmentalized inside vacuolar compartments within pollen tubes early in both compatible and incompatible pollination, and S-RNases are released from these compartments later only during incompatible pollination (Goldraij et al. 2006).

22.3.2.2 Male Determinant of SI

Sequence analysis of the *S*-locus of *Antirrhinum hispanicum* (snapdragon), a member of the family Scrophulariaceae, has identified the existence of a gene encoding a pollen- and tapetum-specific protein with an F-box domain, and this protein was named *Antirrhinum hispanicum S*-locus F-box protein (AhSLF; Lai

et al. 2002). At first, the function of AhSLF as a male determinant was questioned because of the lack of polymorphisms between AhSLF alleles. However, the findings that the *S*-locus F-box proteins of *Prunus mume* (Japanese apricot) were expressed in pollen and exhibited haplotype-specific diversity (78–81% amino acid identity) strongly support the idea that SLF is the male determinant of SI (Entani et al. 2003). This interpretation was also supported by the further identification of the F-box proteins in *P. dulcis* (almond), *P. avium* (sweet cherry), and *P. cerasus* (sour cherry; Lai et al. 2002; Ushijima et al. 2003; Yamane et al. 2003). The F-box proteins identified in *P. mume* were named PmSLF, and those identified in *P. dulcis*, *P. avium*, and *P. cerasus* were named S-haplotype-specific F-box (SFB). Immunocytochemistry and western blot analysis have shown that AhSLF proteins were present in the cytoplasm and peripheral part of the endoplasmic reticulum of pollen tubes (Wang and Xue 2005). Alignment of *Prunus* SLF/SFB amino acid sequences revealed that SLFs/SFBs contain a conserved N-terminus F-box, and two C-terminus hyper-variable regions (HVa and HVb; Ushijima et al. 2003; Kao and Tsukamoto 2004).

In *Nicotiana* and *Petunia*, analyses of pollen-part self-compatible mutants revealed that most of these mutants possess duplications of the entire, or of a part of the *S*-locus that includes the *SLF* region (Golz et al. 2001; Tsukamoto et al. 2005). Interestingly, plants with a mutation that causes the deletion of *SLF* could not be recovered. These results provide support for the function of SLF in the SI response in *Nicotiana*, and suggest that SLF is essential for the viability of pollen tubes, probably because of its antagonistic action to the S-RNase. A different scenario, however, might take place in S-RNase based GSI of *Prunus*, where induced or natural mutations that lead to the loss of SLF/SFB confer self-compatible phenotype on *P. avium* and *P. mume* (Ikeda et al. 2004; Ushijima et al. 2004; Sonneveld et al. 2005).

The function of SLF in SI response was also confirmed by a carefully executed transformation experiment in *Petunia inflata*. The S_2 allele of PiSLF was expressed in the pistil of S_1S_1, S_1S_2 and S_2S_3 plants, and the result from self-pollination showed that the breakdown of SI occurred in S_1 or S_3 pollen carrying the transgene S_2 allele, but did not occur in the S_2 pollen carrying the S_2 allele (Sijacic et al. 2004). Duplication of the *F-box* gene was also found to associate with the breakdown of pollen SI function in a naturally occurring self-compatible accession of *Petunia axillaris* (Tsukamoto et al. 2005). These results explain the phenomenon of heteroallelic pollen effect where SI function is lost in the diploid pollen of a tetraploid plant (ex. $S_1S_1S_2S_2$) expressing two different *S*-haplotypes (ex. S_1S_2), but not those expressing two copies of the same haplotype (ex. S_1S_1 or S_2S_2; de Nettancourt 1997). This experimental evidence strongly implicates SLF as the male determinant of the S-RNase based GSI system.

Proteins with the F-box domain are known to function as a substrate-recognition component of a major type of an E3 ubiquitin ligase called the Skp-Cullin-F-box (SCF) complex. The SCF complex works together with the ubiquitin-activating enzyme (E1) and the ubiquitin-conjugating enzyme (E2) to target proteins for degradation by a 26S proteasome (Risseeuw et al. 2003). The fact that the male

determinant is a member of the F-box protein family implies the involvement of the ubiquitination pathway, and most likely, the ubiquitin-mediated protein degradation pathway in the S-RNase based GSI system.

22.3.2.3 Interaction Between Male and Female SI Determinants

Based on the nature of the male and the female determinants described above, and the observation that pollen tube rRNAs were degraded 12 to 48 h after incompatible pollination (McClure et al. 1990), it is likely that SLFs/SFBs interact with S-RNases and target these for degradation, in so doing protecting pollen tubes from the S-RNase cytotoxic effect. Consistent with this hypothesis, an increase in stylar protein ubiquitination, and a decrease in the amount of S-RNase could be detected after compatible pollination in *Antirrhinum* (Qiao et al. 2004). The interaction between the SLF and the S-RNase was also demonstrated in the same report, albeit in a non-*S*-specific manner (Qiao et al. 2004). Different from the data obtained from *Antirrhinum*, most of the S-RNase population remained stable at 36 h after compatible pollination in *Nicotiana* (Goldraij et al. 2006), and an in vitro binding assay showed that SLF interacts more strongly to the non-self S-RNases than to the self S-RNases in *Petunia* (Hua and Kao 2006). Results from domain-swapping experiments suggested that *Petunia* SLF has a conserved S-RNase binding domain (SBD), and variable domains that conversely regulated interaction between SBD and S-RNase (S-RNase binding regulating domain, SBRD; Hua et al. 2007). It was suggested that SBD confers strong interaction between any given SLF and a domain common to all S-RNase, while allele-specific interaction between SBRD and S-RNase weakens the SBD-S-RNase interaction.

22.3.3 Other Components of S-RNase Based GSI

Although a conclusion has not yet been reached for the functional relationship between the SLF/SFB and the S-RNase, the identification of other components that also function in this GSI system might provide information that can help clarify this relationship.

In an attempt to identify such components, a differential screen was performed to identify stylar genes that are expressed in the self-incompatible *N. alata*, but not in its closely related self-compatible *N. plumbaginifolia*. A gene named *HT-B* was identified by this screen, and was found to encode a protein with unknown function that contains a stretch of 20 Asn and Asp residues (the ND domain; McClure et al. 1999; Cruz-Garcia et al. 2003). A biochemical approach identified a 120-kDa glycoprotein of stylar ECM that directly interacted with S-RNase in an S-RNase affinity column (Cruz-Garcia et al. 2005). Both HT-B and 120K proteins are native to the stylar ECM, and are transported into pollen tubes together with S-RNases. The involvement of HT-B and 120K in the SI response was confirmed in transgenic

experiments in which the expression of antisense *HT-B* in *Nicotiana* and *Solanum*, and of the RNAi *120K* in *Nicotiana* resulted in the breakdown of pistil SI function (McClure et al. 1999; O'Brien et al. 2002; Hancock et al. 2005). Recent findings have revealed that, in *Nicotiana*, HT-B degradation occurred in cross pollen tubes, and that 120K co-localized with vacuolar compartments within which S-RNases reside (Goldraij et al. 2006). Though the exact function of HT-B and 120K in the SI response is still not clear, S-RNases were detected in pollen tubes growing in the styles of both the *HT-B* antisense and the *120K* RNAi plants, and self-compatible phenotypes in these plants resulted from a prolonged compartmentalization of S-RNases (Goldraij et al. 2006). Therefore, HT-B and 120K most probably function to facilitate the event(s) that involve compartmentalization of S-RNases within the pollen tube.

A few components of the GSI pathway from the pollen tube were also identified. PhSBP1 and PiSBP1 from *Petunia*, and ScSBP1 from *Solanum*, all of which are RING-HC proteins that might function as an E3 ubiquitin ligase, were identified by yeast two-hybrid screens as proteins that interact with S-RNase (Sims and Ordanic 2001; O'Brien et al. 2004; Hua and Kao 2006). PhSBP1 interacted with S-RNase in a non-*S*-specific manner, and ScSBP1 interacted with the conserved sequence located between the HVa and HVb regions of S-RNase. In addition to its interaction with S-RNase, PiSBP1 was also found in a complex with PiSLF, PiCUL1-G, and an E2 ubiquitin-conjugation enzyme, but not with *Arabidopsis* SKP proteins. These data point to the possibility that PiCUL1-G, PiSBP1, and PiSLF each functions as a component of an unconventional SCF complex usually composed of four subunits, SKP1, CUL1, F-box protein, and a RING-finger protein (Hua and Kao 2006). However, in *Antirrhinum*, AhSSK1, a SKP1-like protein, was identified as a protein that interacted with AhSLF in a yeast two-hybrid screen. AhSSK1 was also reported to function as a protein that bridged AhSLF to a CUL1-like protein in an in vitro pull-down assay (Huang et al. 2006). Moreover, AhSLF was found to interact with *Antirrhinum* proteins that cross-reacted with antibodies against *Arabidopsis* ASK1 and CUL1, both of which are known components of the SCF complex (Qiao et al. 2004). These data imply that the SCF^{SLF} complex in *Antirrhinum* may contain components different from those of the SCF^{SLF} complex in *Petunia*.

22.3.4 Working Model and Prospects

Significant differences in information were obtained from studies of the S-RNase based GSI system operating in the Solanaceae and Rosaceae. The Rosaceae *SLF/SFB* genes have greater polymorphism than those of the Solanaceae, and *Prunus* SFBs form a separate clade relative to *Petunia* or *Antirrhinum* SLFs (Ushijima et al. 2004). All pollen-part self-compatible mutants identified in the Solanaceae are caused by duplication of *SLF* (Golz et al. 1999, 2001), where as those from Rosaceae are caused by deletion or mutation of *SLF/SFB* (Ushijima et al. 2004; Sonneveld et al. 2005). Heteroallelic pollen (pollen expressing two different

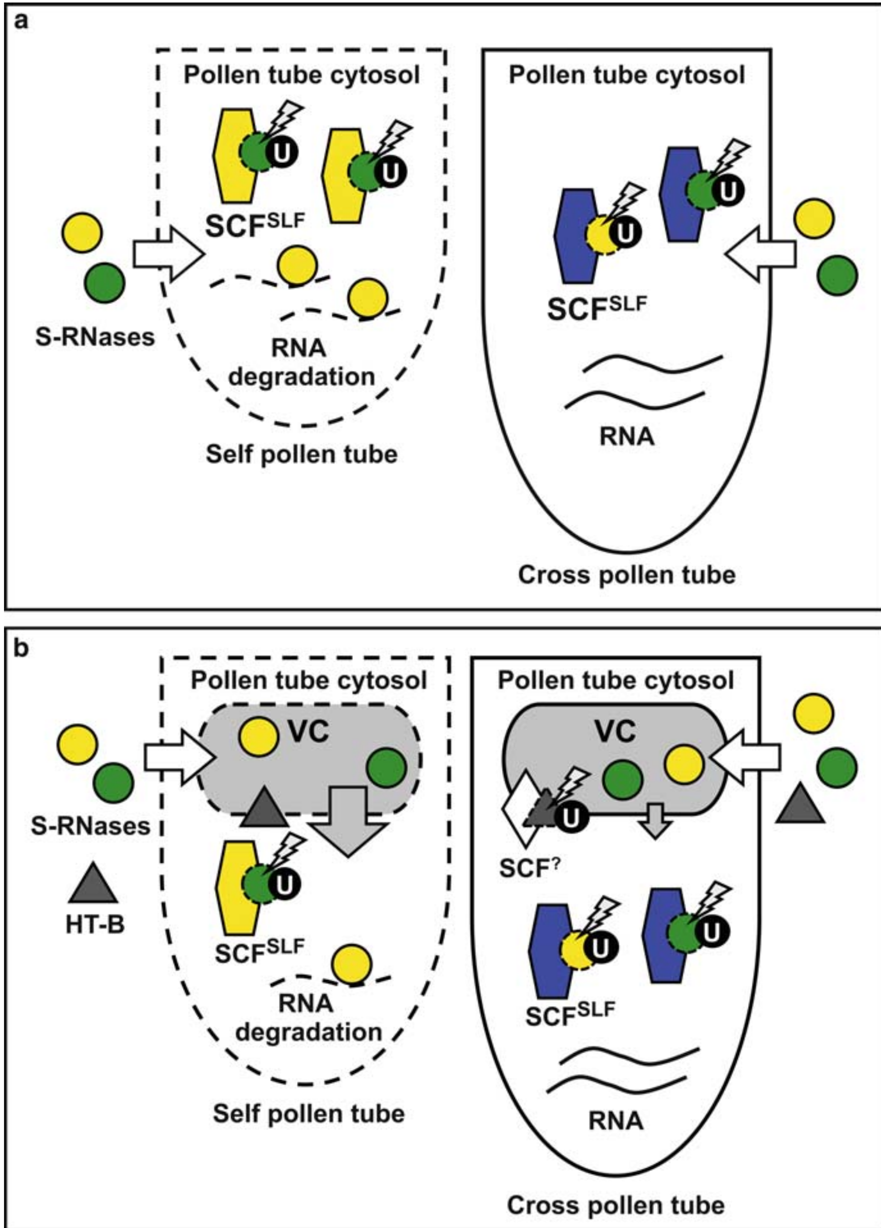

Fig. 22.3 Two hypothetical models for molecular events leading to the gametophytic self-incompatibility (GSI) response in the Solanaceae. **a** Competitive interaction model based on the results from research in *Petunia*. In this model, S-RNases prefer to interact with non-self SLF on the SCF^{SLF} complex, resulting in ubiquitination (U) and proteasome degradation (⚡) of the non-self S-RNases, and elimination of their cyto-toxicity effect. **b** S-RNase sequestration model based on the results from research in *Nicotiana*. The key events in this model are the sequestration of

S-alleles) failed to function in SI in the Solanaceae (Golz et al. 2001; Sijacic et al. 2004), but heteroallelic pollen of the Rosaceae displayed normal SI function (Hauck et al. 2006). These data together suggest that the mechanisms of SI response differ between these two families.

Different information was also obtained for the mechanisms of S-RNase based GSI in Scrophulariaceae and Solanaceae plants. After compatible pollination, a decrease in the amount of S-RNases was detected in *Antirrhinum*, while degradation of HT-B, but not of S-RNase, was detected in *Nicotiana* (Qiao et al. 2004; Goldraij et al. 2006). In *Petunia*, PiSLF interacts much more strongly with a non-self S-RNase than with a self S-RNase, while in *Antirrhinum*, AhSLF interacts with S-RNase in a non-*S*-specific manner (Qiao et al. 2004; Hua and Kao 2006). Moreover, components of the SCF^{SLF} complex in *Petunia* also seem to differ from those in *Antirrhinum* (Qiao et al. 2004; Hua and Kao 2006). Despite these divergences, the overall consensus in this information is that specificity of SI interaction is controlled by S-RNase and SLF on the female and the male side, respectively, and that ubiquitination and subsequent protein degradation by the 26S proteasome are crucial processes operating during the SI response. Membrane trafficking might be the other crucial process required for the SI response, because in *Nicotiana*, S-RNases were detected inside vacuole-like compartments in both self and cross pollen tubes, and only in a self pollen tube did these compartments later break and release their content (Goldraij et al. 2006).

Although still premature, two hypothetical models for the GSI mechanism can be proposed based on existing data from the Solanaceae family, these being the competitive interaction model, and the S-RNase sequestration model (Fig. 22.3). The competitive interaction model is based on the finding that PiSLF interacts much more strongly with non-self S-RNase than with self S-RNase (Hua and Kao 2006), and on the assumption that interaction between SLF and S-RNase leads to degradation of S-RNases. This model can explain the normal SI response mechanism, the heteroallelic pollen phenomenon, the occurrence of dual-specific S-RNase, and the failure to identify any SLF null mutants or mutants with non-functional SLF in *Nicotiana* and *Petunia*. The S-RNase sequestration model accounts for the large amount of S-RNases taken up by the pollen tube. In fact, the two models complement each other when considering the SI mechanism as a three-step process. In the first step, immediately after up-take into the pollen tubes, S-RNases are compartmentalized (sequestration model), and any non-self S-RNase

Fig. 22.3 (continued) S-RNases into a vacuolar compartment (VC) in both self and cross pollen tubes, the degradation of HT-B in a cross pollen tube, and the release of S-RNases from the VC in a self pollen tube. In this model, S-RNases taken up (*white arrow*) by the pollen tube are sequestered immediately into the VC, and are released from the VC (*large gray arrow*) only in response to an unknown mechanism in the presence of HT-B protein. In a cross pollen tube, an unknown mechanism induces HT-B protein degradation, stabilizing the S-RNase compartment, thereby protecting the cross pollen tube from S-RNase toxicity. Interaction between S-RNase and non-self SLF on the SCF^{SLF} complex is also presented here as a backup mechanism for removal of the non-self S-RNase leaking out from the VC (*small gray arrow*). Different colors represent different *S*-haplotypes of the SCF^{SLF} complexes and the S-RNases

that is accidentally released or leaked into the cytosol will be target for degradation by the SCF^{SLF} complex, which has high affinity to non-self S-RNase. In the second step, the SI response results in the breakdown of S-RNase compartments in the presence of HT-B in a self pollen tube, while S-RNase compartments in a cross pollen tube remain intact as a result of HT-B degradation. In the third step, self S-RNases released from the compartment into the cytosol are not recognized by the SCF^{SLF} complex, and as a result, they are free to degrade RNA of the self pollen tube. Clearly, further investigations are required to verify or disprove these models. Some important questions in need of answers include (1) what is the real target of the $SCF^{SLF/SFB}$ complex, (2) what kind of mechanism controls the specificity of HT-B degradation, and how does S-RNase exert its female-determinant effect on this degradation mechanism, and (3) what are the fates of the HT-B, the S-RNase compartment, and the S-RNase itself within heteroallelic pollen tubes.

22.4 The Use of SI in Breeding Programs

22.4.1 Plant Breeding

Although molecular breeding has become a method of choice for improvement of many crops, conventional breeding still holds potential to confer desirable phenotypes unpredictable for the breeders, and impossible to mimic by molecular breeding methods. It has long been known that hybrid seeds that result from cross-fertilization are superior to self-fertilized seeds. Therefore, conventional breeding programs are designed to generate superior F1-hybrid lines by combining desirable traits of available parent lines. To ensure uniformity of the F1-hybrid population, breeders usually use inbred parent lines that are self-pollinated, and selected for many generations to become near-isogenic lines.

Most of the current economically important crops are self-compatible as a result of pressure from cultivation/domestication by humans. Self-compatible parent lines could complicate the breeding process, because breeders have to find a way to maximize the possibility of cross-pollination among these lines, and to make sure that the harvested seeds are hybrids. Typically, breeders ensure the quality of F1-hybrid seeds by integrating a step to emasculate the female parent line before pollination to eliminate the possibility of self-pollination, or by using a female parent line that lacks the ability to self-pollinate (male-sterile line). The latter method is more appealing, because manual emasculation is tedious and time-consuming, leading to high production costs for commercial F1-hybrid seeds. However, for plants that do not produce nectar, but use insects to assist pollination, it is more efficient to employ self-incompatible lines than male-sterile lines, because the main pollinators for these plants (pollen-collecting bees) rarely visit male-sterile plants. Conventionally, self-incompatible lines are obtained by resynthesis of the line by re-crossing known wild parents (usually self-incompatible plants; Rahman 2005), introgression of the line by backcrossing it with one of the

parents (Gowers 1982), or selection of the line mutated naturally, or aided by biochemical or physical means. Although these conventional methods work well, all involve a long process of crossing and maintaining a large number of plant lines. The F1 hybrid resulting from the cross between two self-incompatible parents, or between a self-compatible male parent and a self-incompatible female parent, results in an F1 hybrid that is also self-incompatible. This is acceptable in the case of vegetable and ornamental crops, but for crops yielding commercial products in the form of seeds (canola, rapeseeds) or fruits (apple, apricot, cherry, pear, plum), self-compatibility is a more desirable trait due to the ease of seed and fruit production. Therefore, a breeding program to produce self-compatible F1 hybrids is also in demand. In general, the methods to breed self-compatible F1 hybrids involve the use of nuclear or cytoplasmic male-sterile lines, and of the different techniques attached to these to restore male fertility in the F1 hybrid (Havey 2004). Again, the downfall of these methods is the time needed for backcrossing. A faster approach to produce self-incompatible parent lines and self-compatible F1 hybrids is required, especially for fruit crops with a long generation time.

22.4.2 Integration of the SI System into F1-Hybrid Breeding Programs

As mentioned above, there is a demand for self-incompatible parent lines in order to eliminate/reduce contamination of self-seeds in F1-hybrid seed stock, and for self-compatible F1-hybrid lines of seed and fruit crops. Given the present knowledge about the SI systems described in this chapter, self-incompatible and self-compatible lines can now be generated with support from biotechnology and genetic engineering techniques (collectively called SI technology here). SI technology can also be used in combination with conventional breeding programs to shorten the time required. Examples of available technologies and breeding programs to generate these desirable lines, as well as the use of SI technology to facilitate conventional breeding program, are described below.

22.4.2.1 Generation and Maintenance of Inbred Self-Incompatible Parent Lines with SI Technology

Theoretically, the generation of self-incompatible plants from normally self-compatible plants can be achieved by a transgenic plant approach. For example, if the gene responsible for the self-compatible phenotype in a given plant is known, then SI can be reestablished by reintroducing the functional copy of the gene into the self-compatible plant. This process is straightforward, but requires biotechnology techniques including genetic engineering to generate papilla/stigma- or pollen-specific transgene-expression systems and transformation, selection, and tissue culturing. Conversion of self-compatible lines of plants into self-incompatible lines has been demonstrated in *A. thaliana*, the model SSI plant, as mentioned above. For crops in

the genus *Brassica*, although the conventional introgression approach could restore SI function to the normally self-compatible *B. napus* (Goring 1992), the successful generation of self-incompatible plants from self-compatible plants by genetic engineering has not been reported. However, it is evident that SP11/SCR and SRK, when expressed alone, could confer their haplotype-specific determinant activity on transformed self-incompatible *Brassica* plants (described above). Therefore, it can be contemplated that expression of both genes together in a self-compatible *Brassica* plant defective in these genes will result in the conversion of self-compatible into self-incompatible phenotypes.

While the self-incompatible phenotype is useful for the production of F1-hybrid seed, the maintenance of self-incompatible lines from one generation to the next can be troublesome. To date, stocks of self-incompatible lines can be maintained by tissue culture or bud pollination techniques, or by techniques to transiently break down the SI barrier. In plants with the SSI system, the SI barrier can be transiently overcome by treating the pistil with NaCl solution (Monteiro 1988; Kučera et al. 2006) or CO_2 gas (Nakanishii et al. 1969; Kučera et al. 2006), while in plants with the S-RNase based GSI system, a commercially available S-RNase blocker (a mixture of divalent Zn and Cu ions) is used (Kim et al. 2001).

22.4.2.2 Generation of Self-Compatible F1 Hybrids with SI Technology

Breeding programs for seed or fruit crops usually incorporate systems to confer self-compatible phenotypes to F1-hybrid plants. This can be achieved easily when SI technology is incorporated into the breeding system. Mutations that compromise the activity of female SI determinants, or antisense expression/co-suppression of the female SI determinant genes, always result in the breakdown of the SI response (see above). This observation also holds for commercial crops such as almonds (Bošković et al. 2007) and apple (Broothaerts et al. 2004). Therefore, to generate self-compatible F1 hybrids, the cross can be made between a self-incompatible female parent and a self-compatible male parent expressing an antisense construct, or a construct expressing a dominance-negative form of the female SI determinant (SRK for plants with SSI system, or S-RNase for plants with S-RNase based GSI system; Canadian patent application number 2279496). Because disruption of the modifier genes, e.g., *MLPK*, *ARC1*, and *HT-B*, can also contribute to the breakdown of SI, manipulation of the expression or of the function of these modifiers might also be useful for breeding programs of self-compatible F1 hybrids. For S-RNase based GSI plants, however, applying the commercially available S-RNase blocker (cf. above) to self-incompatible F1-hybrid plants might be more practical than starting a program to breed a new line of self-compatible F1 hybrids.

22.4.2.3 Supporting Role of SI Technology in Conventional Breeding Programs

The availability of gene sequences for the *S*-locus has made it possible for breeders to molecularly identify the *S*-haplotype of the parent or the F1-hybrid plants without having to perform time- and resource-consuming test crosses. This again is especially beneficial for breeding programs of plants with long generation cycles. Molecular screening [usually PCR (polymerase chain reaction) based] for the presence of a specific *S*-locus can be performed using small amounts of leaf tissues from seedlings. This eliminates the requirement to cultivate the unwanted plants to maturity. Molecular *S*-haplotyping is performed in Brassicaceae plants by PCR amplifying the *S*-genes, followed by restriction-fragment size and sequence analyses of the PCR products (Brace et al. 1994; Nishio et al. 1996, 1997; Park et al. 2002; Mohring et al. 2005; Okamoto et al. 2007; Zhang et al. 2007), and in members of the Solanaceae and Rosaceae by reverse-transcription PCR of the S-RNase transcripts, followed by size and sequence analyses (Richman et al. 1996; Goldway et al. 2007).

22.5 Conclusions

SI systems in flowering plants have been the topic of interest for researchers, breeders, and naturalists for more than 100 years (Darwin 1876). In recent years, significant progress has been made toward the understanding of the SI systems described in this chapter, as well as the SI systems in the families Papaveraceae (i.e., poppy) and Poaceae (grasses), reviewed in Bosch and Franklin-Tong (2008) and Yang et al. (2008). Equipped with the knowledge of SI systems, a new generation of breeders have begun to incorporate SI technology into their breeding programs. As the advances in plant molecular biology and biotechnology continue to grow, it is promising that more questions in self-incompatibility will be answered, and knowledge gained will be transferred to breeders for crop improvement.

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